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(54) Title: *NBC*, A GENE THAT ENCODES A MEMBER OF THE BICARBONATE TRANSPORTER FAMILY OF PROTEINS

(57) Abstract

The present invention provides the nucleotide sequence of nucleic acid molecules that encode members of the Sodium Bicarbonate Cotransporter (NBC) family of proteins. Based on this disclosure, the present invention provides isolated NBC protein, isolated NBC encoding nucleic acid molecules, methods of isolating nucleic acid molecules that encode other members of the NBC family of proteins, methods for identifying agents that agonize or antagonize NBC activity, methods of using agents that agonize or antagonize NBC activity to modulate biological and pathological processes, and methods of assaying for NBC activity.

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# *NBC*, A GENE THAT ENCODES A MEMBER OF THE BICARBONATE TRANSPORTER FAMILY OF PROTEINS

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## FIELD OF THE INVENTION

The present invention relates to the fields of extra and intracellular pH and cellular transport of sodium ions ($Na^+$), bicarbonate ions ($HCO^-_3$) and water. The invention relates specifically to the identification of a novel gene, tentatively named *NBC* (Na, Bicarbonate Cotransporter). *NBC* encodes a protein, NBC, that is a member of the Bicarbonate Transporter family of proteins.

## BACKGROUND OF THE INVENTION

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

### Importance of $pH_i$

Almost all cellular events are pH sensitive (Busa, W. B. et al., *Biochem. Biophys. Acta* (1978) 515:239-302). The rate-limiting enzyme in glycolysis, phosphofructokinase, and a critical ribosomal protein, S6, both go from being almost fully "off" to fully "on" with a pH shift only ~0.1. Proliferation in response to growth factors requires a sufficiently alkaline $pH_i$ and the list of major ion channels with substantial pH sensitivity is ever growing. Because so many key processes are $pH_i$ sensitive, cells have evolved acid-base transporters, localized in the plasma membrane, to regulate $pH_i$. In turn, acid-base transporters are under control of hormones, growth factors or other signals, such as cell volume. In most cells, the most powerful of these acid-base transporters carry $HCO^-_3$.

**$pH_i$ Regulation**

Cells regulate their $pH_i$ using a classic pump-leak mechanism. The "pumps" in this case are acid extruders (i.e., active transporters that tend to increase $pH_i$), such as the vacuolar-type $H^+$ pump and the Na-H exchanger. The "leaks" are acid loaders, passive mechanisms that tend to decrease $pH_i$.

**$HCO^-_3$ reabsorption by the kidney**

One of the major functions of the renal proximal tubule is to reclaim $HCO^-_3$ that has been filtered in the glomerulus. This reabsorption of $HCO^-_3$ from the proximal-tubule lumen to the blood helps to maintain an appropriately high [$HCO^-_3$] in the blood, and thus helps to stabilize blood pH. Failure to reabsorb sufficient $HCO^-_3$ would not only lead to the loss of $HCO^-_3$ in the urine, but also to the loss of $Na^+$ (which normally accompanies reabsorbed $HCO^-_3$) and osmotically obligated water. Thus, the consequences of reduced $Na^+$ reabsorption would be metabolic acidosis and volume depletion.

As shown in Figure 1, $HCO^-_3$ reabsorption by the proximal tubule is a multistep process. First, $H^+$ is secreted into the lumen by $Na^+/H^+$ exchangers (antiporters) and presumably vacuolar-type $H^+$ pumps (V-type ATPases). Second, this $H^+$ titrates luminal $HCO^-_3$ to $CO_2$ and $H_2O$, a process accelerated by carbonic anhydrase (CA) IV, which is tethered to the extracellular surface of the apical membrane. Third, the newly formed $CO_2$ and $H_2O$ enter the proximal-tubule cell. Fourth, cytoplasmic CA II accelerates the regeneration of $H^+$ and $HCO^-_3$. Finally, this $HCO^-_3$ exits the proximal-tubule cell across the basolateral membrane (BLM), completing the movement of $HCO^-_3$ from lumen to blood.

In the portion of the proximal tubule furthest downstream from the glomerulus, the S3 segment, the efflux of $HCO^-_3$ across the basolateral membrane is mediated both by a $Cl^-/HCO^-_3$ exchanger and an electrogenic $Na^+$:$HCO^-_3$ cotransporter (Nakhoul, N.L., et al. *Am J Physiol* (1990) 258:F371-F381). However, in more proximal segments of the proximal tubule (S2 and S1 segments), where the bulk of $HCO^-_3$ is in fact reabsorbed, the relative contribution of $Cl^-/HCO^-_3$ exchange decreases, and that of electrogenic $Na^+$:$HCO^-_3$ cotransport increases (Kondo, Y. et al. *Pflügers Arch* (1987) 410:481-486). All told, the proximal tubule reabsorbs some 85% of the filtered $HCO^-_3$ and 70% of the filtered $Na^+$. The electrogenic $Na^+$:$HCO^-_3$ cotransporter carries the vast majority of this $HCO^-_3$, and about 5%

of the $Na^+$.

**The electrogenic $Na^+$:$HCO^-_3$ cotransporter**

First described by in the proximal tubule of the salamander (Boron, W.F. et al. *J Gen Physiol* (1983) 81:53-94), electrogenic $Na^+$:$HCO^-_3$ cotransporters have subsequently been studied in several epithelial cell lines (Jentsch, T.J., et al. *J Membrane Biol* (1984) 81:189-204; Jentsch, T.J., et al. *J Biol Chem* (1985) 260:15554-15560), intact rat and rabbit proximal tubules (Alpern, R.J. *J Gen Physiol* (1985) 86:613-636; Sasaki, S., *Am J Physiol* (1987) 252:F11-F18), as well as in a number of other cell types (Deitmer, J.W. et al. *J Physiol Lond* (1989) 411:179-194; Gleeson, D., et al. *J Clin Invest* (1989) 84:312-321; Fitz, J.G., et al. *Am J Physiol* (1989) 256:G491-G500; Weintraub, W.H. et al. *Am J Physiol* (1989) 257:G317-G327; Rajendran, V.M., et al. *J Clin Invest* (1991) 88:1379-1385; M. O. Bevensee and W. F. Boron, submitted; M. O. Bevensee, M. Apkon and W. F. Boron, submitted). The four hallmarks of the renal $Na^+$:$HCO^-_3$ cotransporter are its electrogenicity, $Na^+$ dependence, $HCO^-_3$ dependence, and blockade by disulfonic stilbene derivatives such as DIDS. Probably the most reliable assay for detecting the electrogenic $Na^+$:$HCO^-_3$ cotransporter in intact cells is to remove extracellular $Na^+$. This maneuver causes an abrupt positive shift (i.e., depolarization) in the cell membrane voltage ($V_m$). In the absence of $HCO^-_3$, in presence of DIDS or in cells lacking the cotransporter, removing $Na^+$ generally causes a small negative shift in $V_m$, reflecting the normal $Na^+$ conductance of the cell. In the presence of a functional electrogenic $Na^+$:$HCO^-_3$ cotransporter, $Na^+$ removal also causes a slower fall in intracellular pH ($pH_i$), as well as a DIDS-sensitive fall in $[Na^+]_i$.

The electrogenicity of the cotransporter implies that each $Na^+$ moves with two or more $HCO^-_3$ or $HCO^-_3$ equivalents (e.g., $CO^{-2}_3$). The $Na^+$:$HCO^-_3$ stoichiometry has not been directly measured. However, it has been deduced by Soleimani, M., et al. *J Clin Invest* (1987) 79:1276-1280 on the basis of thermodynamic data obtained from membrane vesicles prepared from the basolateral membranes of rabbit proximal tubules. The principle underlying these experiments is that the net direction of cotransport depends on the stoichiometry, as well as the $Na^+$ and $HCO^-_3$ electrochemical gradients. Varying these gradients while measuring the net direction of transport, the authors concluded that the renal electrogenic $Na^+$:$HCO^-_3$ cotransporter has a $Na^+$:$HCO^-_3$ stoichiometry of 1:3. Further work

(Soleimani, M. et al. *J Biol Chem* (1989) 264, 18302-18308) was consistent with the hypothesis that the renal electrogenic $Na^+$:$HCO^-_3$ cotransporter has binding sites for 1 $Na^+$, 1 $HCO^-_3$ and 1 $CO^{-2}_3$, and that sulfite ($SO^{-2}_3$) can replace $CO^{-2}_3$ at the $CO^{-2}_3$ site (see Figures 1-3).

**The physiological family of $HCO^-_3$ transporters**

The $HCO^-_3$ transporters shown in Figure 3 might be thought of as a functional family of transporters that may or may not be related at the molecular genetic level. The renal electrogenic $Na^+$:$HCO^-_3$ cotransporter, with a $Na^+$:$HCO^-_3$ stoichiometry of 1:3, is one of as many as four $Na^+$-coupled $HCO^-_3$ transporters, as well as a $Cl^-/HCO^-_3$ exchanger and a $K^+$:$HCO^-_3$ cotransporter. There is at least one, and perhaps two, other $Na^+$:$HCO^-_3$ cotransporters that normally mediate a net influx, rather than a net efflux, of $HCO^-_3$ equivalents (see Boron, W.F. et al., *Kidney Int* (1989) 36:392-402). That is, they are acid extruders rather than acid loaders. In invertebrate glial cells (Deitmer, J.W. et al. *J Physiol Lond* (1989) 411:179-194), mammalian astrocytes and other cells, an electrogenic $Na^+$:$HCO^-_3$ cotransporter mediates a net influx of $Na^+$ and $HCO^-_3$. Based on analyses of electrochemical gradients of $Na^+$ and $HCO^-_3$, it is believed that such acid-extruding electrogenic $Na^+$:$HCO^-_3$ cotransporters have a 1:2 stoichiometry. Thus, it is the stoichiometry of the cotransporter, as well as the electrochemical gradients of the transported ions, that determines the net direction of transport, and whether the cotransporter behaves as an acid loader (as is generally the case with a 1:3 stoichiometry) or an acid extruder (as is generally the case with a 1:2 stoichiometry). In addition to cotransporters with stoichiometries of 1:3 and 1:2, work by Vaughan-Jones and colleagues (Dart, C. et al. *J Physiol Lond* (1992) 451:365-385) on heart cells suggests that these cells have an electroneutral $Na^+$:$HCO^-_3$ cotransporter with a $Na^+$:$HCO^-_3$ stoichiometry of 1:1. Camilion de Hurtado, M.C., et al. *J Mol Cell Cardiol* (1995) 27:231-242, however, concluded that this cotransporter is an electrogenic 1:2 $Na^+$:$HCO^-_3$ cotransporter.

The $Na^+$-driven $Cl^-/HCO^-_3$ exchanger is the major acid extruder in a wide variety of animal cells. In contrast to the two electrogenic $Na^+$:$HCO^-_3$ cotransporters, the $Na^+$-driven $Cl^-/HCO^-_3$ exchanger is electroneutral and has an absolute requirement for $Cl^-$. Like the 1:2 and 1:1 $Na^+$:$HCO^-_3$ cotransporters, the $Na^+$-driven $Cl^-/HCO^-_3$ exchanger normally moves $HCO^-_3$ into cells. Aside from the work described here on the cloning of the 1:3 electrogenic

$Na^+$:$HCO^-_3$ cotransporter, there is no published molecular information on any of the $Na^+$-coupled $HCO^-_3$ transporters.

The $Cl^-/HCO^-_3$ exchanger, sometimes referred to as the $Na^+$-independent $Cl^-/HCO^-_3$ exchanger to distinguish it from the $Na^+$-driven $Cl^-/HCO^-_3$ exchanger, was the first $HCO^-_3$ transporter to be described. There is now considerable information on the molecular biology and biochemistry of $Cl^-/HCO^-_3$ exchangers. The first of these to be cloned was the band-3 protein of red blood cells, also known as AE-1 (for Anion Exchanger) (Kopito, R.R. et al. *Nature* (1985) 316:234-238). AE-1 plays a major role in $CO_2$ carriage by erythrocytes. Two related $Cl^-/HCO^-_3$ exchangers, AE-2 and AE-3, are present in a wide variety of non-erythroid cells. Because they normally mediate the exchange of extracellular $Cl^-$ for intracellular $HCO^-_3$, AE-2 and AE-3 function as intracellular acid loaders.

For those interested in understanding the mechanisms of pH regulation, or in developing agents for modulating intracellular and extracellular pH, $Na^+$, and $HCO^-_3$, there is a need for substantial quantities of pure proteins representing various members of the Bicarbonate Transporter (BT) superfamily of proteins. There is also a need for diagnostic tools for use in determining transporter activity. To this end, the present invention provides isolated nucleic acid molecules that encode various members of the BT superfamily of proteins which are useful in producing the transporter for use in binding/modulation assays and for use in diagnostic screens.

## SUMMARY OF THE INVENTION

The present invention is based, in part, on the isolation and identification of nucleic acid molecules that encode proteins that are members of the 1:3 $Na/HCO_3$ family of proteins, hereinafter *NBC* genes or *NBC*, or NBC encoding nucleic acid molecules (Na Bicarbonate Cotransporter). In the Examples, an NBC encoding nucleic acid molecule was isolated from salamander, the *sNBC* gene, from rat, the *rNBC* gene, and from humans, the *hNBC* gene. Accordingly, the present invention provides isolated nucleic acid molecules that encode an NBC protein, and in particular an NBC proteins from salamanders, rats and humans. Such nucleic acid molecules can be in an isolated form, or can be operably linked to expression-control elements or vector sequences.

The present invention further provides methods of identifying nucleic acid

molecules that encode other members of the NBC family of proteins. Specifically, nucleic acid sequences that encode the NBC proteins herein described can be used as probes, or to generate PCR primers, in methods that can be used to identify nucleic acid molecules that encode other members of the NBC family of proteins.

The present invention further provides methods of identifying other members of the Bicarbonate Transporter (BT) superfamily of proteins. Specifically, the herein described nucleic acid sequences that encode the NBC proteins can be used as probes, or to generate PCR primers, in methods to identify nucleic acid molecules that encode other members of the BT superfamily of proteins. These include members of the 1:2 $Na/HCO_3$ cotransporter family, the 1:1 $Na/HCO_3$ cotransporter family, the $K/HCO_3$ cotransporter family and the Na driven $Cl\text{-}HCO_3$ exchanger family of proteins.

The present invention further provides antibodies that bind to the NBC proteins of the present invention. Cloning of several *NBC* genes, a salamander *NBC* gene, a rat *NBC* gene and a human *NBC* gene and the elucidation of the entire amino acid sequence of several NBC proteins allows one to select epitopes of the NBC protein for generating novel anti-NBC antibodies. Such antibodies can be either polyclonal or monoclonal. Anti-NBC antibodies can be used in a variety of diagnostic formats and for a variety of therapeutic methods.

The present invention further provides methods for determining whether binding partners of the NBC proteins exist and isolating the binding partners if present. NBC binding partners are isolated using the NBC protein, or a fragment thereof, as a capture probe. Alternatively, NBC-encoding nucleic acid molecules can be used as bait in the yeast two-hybrid system to screen an expression library and identify genes that encode proteins that bind to an NBC protein. Binding partners isolated by these methods are useful in preparing antibodies and also serve as targets for drug development.

The present invention further provides methods to identify agents that can block or modulate the activity of an NBC protein. Specifically, an agent can be tested for the ability to modulate the activity of an NBC protein by identifying agents that bind to an NBC protein or agents that block, reduce, activate or otherwise modulate the activity of an NBC protein. Such methods are accomplished by contacting NBC, or a fragment thereof, with a test agent, optionally in the presence of a binding partner, and determining whether the test

agent binds to the NBC protein and/or blocks or reduces the binding of the NBC protein to the binding partner. Such methods can be used to identify agonist and antagonists of an NBC protein.

The present invention further provides methods for reducing or increasing the activity of an NBC protein. Specifically, agonists of an NBC protein can be used to stimulate the activity of the NBC protein whereas antagonists of an NBC protein can be used to decrease the activity of an NBC protein. Such methods provides means for controlling and/or altering extra- or intracellular pH, $Na^+$ concentration and $HCO^-_3$ concentration, as well as the concentrations of others ions, the transport of which is linked to the transport of $Na^+$ (e.g., $Ca^{++}$) or of $HCO^-_3$ (e.g., $Cl^-$).

The present invention further provides methods of regulating the expression of an NBC-encoding nucleic acid molecule within a cell. Expression of an NBC-encoding nucleic acid molecule within a cell can be regulated so as to stimulate or inhibit the production of an NBC protein. Genetic manipulation within an organism can be used to alter the expression of an NBC gene or the production of an NBC protein in an animal model. For example, an NBC gene can be altered to correct a genetic deficiency; peptide modulators of NBC activity can be produced within a target cell using genetic transformation methods to introduce a modulator encoding nucleic acid molecules into a target cell; etc. The use of nucleic acids for antisense and triple helix therapies and intraventions are expressly contemplated.

Agonists, antagonists and altering NBC expression can be used as means for treating/modulating biological and pathological processes that require an NBC protein. For example, agonists and methods that reduce or stimulate NBC protein production can be used to alter intracellular or intracellular pH, $Na^+$ and/or $HCO^-_3$ concentration.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Model of $HCO^-_3$ reabsorption by the mammalian renal proximal tubule. The renal electrogenic $Na^+$:$HCO^-_3$ cotransporter, which has a $Na^+$:$HCO^-_3$ stoichiometry of 1:3, is the major route for transferring $HCO^-_3$ from the proximal-tubule cell to the blood and is responsible for moving as much as ~90% of the $HCO^-_3$ transported by the proximal tubule, and ~75% of the $HCO^-_3$ filtered by the glomeruli. The cotransporter is also responsible for

directly moving ~5% of the $Na^+$ from cell to blood. As such, the cotransporter functions as an auxiliary $Na^+$ pump. The steady-state $pH_i$ of proximal-tubule cells in $CO_2/HCO^-_3$ is ~7.1 - ~7.5, being more alkaline near the glomerulus. The pH of the luminal fluid falls from 7.4 at the glomerulus to ~6.6 at the end of the proximal tubule. The membrane voltage across the basolateral membrane is more negative than -60 mV (Boulpaep, E.L., *Kidney Int.* (1976) 9, 88-102). The lumen is a few mV negative with respect to the blood.

Figure 2. Model of the renal electrogenic $Na^+:HCO^-_3$ cotransporter, which has a $Na^+:HCO^-_3$ stoichiometry of 1:3. Kinetic evidence (Soleimani, M. and Aronson, P.S., *J. Biol. Chem.* (1989) 264, 18302-18308) suggests that there may be separate binding sites for $Na^+$, $HCO^-_3$ and $CO^{-2}_3$, with $SO^{-2}_3$ substituting for $CO^{-2}_3$ at the $CO^{-2}_3$ site.

Figure 3. Major $HCO^-_3$ transporters in animal cells. Three of these transporters normally cause $pH_i$ to decrease: the $Cl^-/HCO^-_3$ exchanger, the 1:3 electrogenic $Na^+:HCO^-_3$ cotransporter, and the $K^+:HCO^-_3$ cotransporter. Three others normally cause $pH_i$ to increase: the $Na^+$-driven $Cl^-/HCO^-_3$ exchanger, the 1:2 electrogenic $Na^+:HCO^-_3$ cotransporter and the 1:1 electroneutral $Na^+:HCO^-_3$ cotransporter.

Figure 4. Injecting RNA and assaying it for activity of an electrogenic $Na^+:HCO^-_3$ cotransporter. First, poly(A)$^+$ RNA (or cRNA synthesized from NBC cDNA) is microinjected into a *Xenopus laevis* oocytes. After several days, the oocyte is impaled with pH and voltage electrodes in order to monitor the $pH_i$ and $V_m$ changes characteristic of the electrogenic $Na^+:HCO^-_3$ cotransporter.

Figure 5. A, Amino acid sequence of NBC. A 4,078 bp cDNA encoding the renal electrogenic Na / Bicarbonate Cotransporter (NBC) was isolated by screening a size-selected (3.5-5.0 kb) *Ambystoma*-kidney cDNA library for electrogenic cotransport of $Na^+$ and HCO3 (see Figure 8 for assay). The cDNA has a poly(A)$^+$ tail, an open reading frame from nucleotides 33 to 3107 encoding a 1025 amino acid protein, and a large 3'-untranslated region (~1 kb, not shown). Rescreening the library led to the identification of an additional 186 nucleotides at the 5' end, resulting in an additional ten amino acids 5' to the original

start methionine, for a total length of 1035 amino acids. A DIDS-binding motif of KL(X)K, where X is I, V, or Y, is present in the AE family (Kopito, R.R., et al., Cell (1989) 59, 927-937). In the region homologous to the AE consensus DIDS-binding motif KL(X)K, NBC has the sequence KMIK (558-561), suggesting a K(Y)(X)K motif, where Y is M or L. NBC contains the AE consensus DIDS-binding motif, KLKK, at 768-771. B, Hydropathy plot of NBC. Ten membrane spanning regions (i.e., 10 hydrophobic regions) were predicted from the primary amino acid sequence using a Kyte-Doolittle algorithm (18 amino acids window size). A large exofacial loop (5,6-EFL) is predicted between MSDs 5 and 6. The AEs also have 10 hydrophobic regions, however, each of the latter two (9 and 10 for NBC) are extensive enough to span the lipid bilayer twice (Reithmeier, R.A.F. Curr Opin Struct Biol (1993) 3, 515-523), consistent with up to 12 MSDs. Others have suggested as many as 14 MSDs (Reithmeier, R.A.F. Curr Opin Struct Biol 3, 515-523 (1993); Alper, S.L. Annu. Rev. Physiol. (1991) 53, 549-564). C, Dendrogram showing relationships among sNBC, and three most homologous members of the AE family (GenBank Accession #'s: S03074, S21086, A42497). The divergence is indicated by the total length of the line from one sequence to another. Using DNAstar (Lasergene, Madison, WI) NBC is 35%, 33%, and 34% similar to AE3, AE2, and AE3, respectively. D, Membrane model of NBC protein. Putative MSDs are indicated by numbered rectangles. DIDS-binding motifs are indicated as green diamonds. Of 8 consensus N-linked glycosylation sites, only 4 (N-glyc in red) are predicted to be extracellular (amino acids 591, 596, 609, and 617; not 34, 160, 209 and 496). Ser 982, predicted to be intracellular, is the only consensus protein kinase A (PKA, pink triangle) site. Of the 15 consensus sites for protein kinase C (PKC, purple circles), 12 are predicted to be intracellular (Ser 18, Thr 85, Thr 173, Thr 206, Ser 219, Thr 345, Ser 357, Thr 394, Thr 706, Ser 810, Ser 1000, Ser 1020), and 3 to be extracellular (Ser 626, Thr 671, Thr 678). D, Membrane model of NBC protein. Because hydropathy plots of NBC and the AEs suggest a similar structure, our NBC model is based on those for the AEs (Reithmeier, R.A.F. *Curr Opin Struct Biol* (1993) 3, 515-523). Putative membrane-spanning domains are indicated by numbered rectangles. Of 8 consensus N-linked glycosylation sites, only 4 (N-glyc) are predicted to be extracellular (amino acids 591, 596, 609, and 617; not 34, 160, 209 and 496). Ser 982, predicted to be intracellular, is the only consensus protein kinase A (PKA, pink triangle) site. Of the 15 consensus sites for protein

kinase C (PKC, purple circles), 12 are predicted to be intracellular (Ser 18, Thr 85, Thr 173, Thr 206, Ser 219, Thr 345, Ser 357, Thr 394, Thr 706, Ser 810, Ser 1000, Ser 1020), and 3 to be extracellular (Ser 626, Thr 671, Thr 678).

Figure 6. High-stringency northern analysis of poly(A)$^+$ RNA from *Ambystoma* tissues probed with $^{32}$P-labeled NBC-cDNA, and exposed for 21 h. After a 10-h exposure (not shown), a 4.1 kb transcript was found in the kidney lanes only. After 21 hours of exposure, or longer (not shown), similar transcripts are seen in bladder, small intestine, large intestine, brain and eye.

Figure 7. Expression-cloning assay in *Xenopus* oocytes. A, Control oocyte injected with water. The external solution (pH 7.5) was switched from ND96 solution ($CO_2/HCO^-_3$ free) to a solution buffered with 1.5% $CO_2$ / 10 mM $HCO^-_3$. $Na^+$ was then removed in the continuous presence of the $CO_2/HCO^-_3$. In water-injected oocytes, removing $Na^+$ from ND96 (not shown) also elicits a hyperpolarization without changing the $pH_i$. B, Oocyte injected with *Ambystoma* kidney poly(A)$^+$ RNA. The $CO_2/HCO^-_3$ was added before the portion of the experiment shown. The protocol was the same as in panel A.

Figure 8. Physiology of NBC clone expressed in oocytes. A, DIDS sensitivity. The external solution was switched from ND96 solution ($CO_2/HCO^-_3$ free) to 1.5% $CO_2$ / 10 mM $HCO^-_3$, at a fixed pH 7.5. $Na^+$ was then removed four times in the continuous presence of the $CO_2/HCO^-_3$. The last two $Na^+$ removals were made in the continuous presence of 200 μM 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS). In the presence of DIDS, the response to $Na^+$ removal was similar to that observed in water-injected oocytes in the absence of DIDS (Figure 7A). B, $HCO^-_3$ dependence. From a resting value of 7.2 to 7.4 in ND96, $pH_i$ decreased to ~7.0 upon adding either 1.5% $CO_2$ / 10 mM $HCO^-_3$ or 10 mM butyrate. $Na^+$ was removed five times, twice in the continuous presence of $CO_2/HCO^-_3$, and three times in the presence of butyrate. The hyperpolarizing response to $Na^+$ removal in butyrate is similar to that observed in water-injected oocytes in the presence of $CO_2/HCO^-_3$ (Figure 7A). C, Changing $[HCO^-_3]_o$ at a fixed $[CO_2]$. Maintaining a $P_{CO_2}$ of 1.5%, we changed $[HCO^-_3]_o$ from 10 to 2 mM (pH: 7.5 to 6.8). After two 0-$Na^+$ pulses, we changed $[HCO^-_3]_o$ from 10 to 32 mM (pH: 7.5 to 8.0). D, NBC currents. The oocyte was voltage-

clamped at-60 mV as we switched from ND96 to a solution buffered with 1.5% $CO_2$ / 10 mM $HCO^-_3$. The ~50 nA outward current elicited by $CO_2/HCO^-_3$ corresponds to a ~50 mV hyperpolarization observed in unclamped cells; the ~50 nA inward current elicited by $Na^+$ removal corresponds to a ~50 mV depolarization observed in unclamped cells. Thus, NBC expression does not significantly change the native oocyte resistance of ~1 MΩ.

Figure 9. Sequence comparison of NBC sequences.

Figure 10. Sequence comparison of two partial length rat-brain NBC-related clones and a portion of rkNBC.

Figure 11. Sequence comparison of a partial length rat-brain NBC-related clone and a portion of rkNBC.

Figure 12. Representation of the NBC cDNA with the location of various DNA oligonucleotide primers.

Figure 13. Sequence of PCR primers.

Figure 14. PAC DNA PCR products using human NBC primers to predict regions for introns.

Figure 15. Sequences for human heart NBC (hhNBC). A. Nucleotide sequence. B. Amino Acid sequence.

Figure 16. N-terminus of hhNBC.

Figure 17. Nucleotide sequence for rat aorta NBC (raNBC).

Figure 18. Nucleotide sequence for NT2-1A.

Figure 19. Sequences for NT2-2A. A. Nucleotide sequence. B. Amino Acid Sequence.

Figure 20. Sequences for S11. A. Nucleotide sequence. B. Amino Acid Sequence.

Figure 21. Western Blot of Crude Membrane Extracts. A fusion antigen of MBP to a fragment of NBC, residues 338-391 (GP3) or residues 928-1035 (GP5), was used to generate polyclonal antisera in guinea pigs. The antisera was used to probe western blots of crude membranes isolated from Ambystoma tigrinum, rat and rabbit.

Figure 22. External $HCO_3$ dependence of rkNBC expressed in *Xenopus* oocyte.

Figure 23. External $HCO_3$ dependence of akNBC expressed in *Xenopus* oocyte.

Figure 24. Effect of $SO_3^=$ and SO4= on rkNBC expressed in *Xenopus* oocyte.

Figure 25. Effect of $SO_3^=$ on DIDS sensitive component of $HCO_3$ -induced $pH_i$ increase in *Xenopus* oocyte expressing rkNBC.

Figure 26. Sequences for human kidney NBC (hkNBC, sometimes designated as hkyNBC). A. Nucleotide sequence. B. Amino Acid Sequence.

Figure 27. Sequences for human pancreas NBC (hpNBC, sometimes called hpanNBC). A. Nucleotide sequence. B. Amino Acid Sequence.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

### I. General Description

As discussed in the Background Section, prior to the present invention, several different classes of Bicarbonate Transporters (BT) were known in the art but were only partially characterized. One class of BT proteins, the 1:3 Sodium Bicarbonate Cotransporters, had been described. However, due to their size and the transmembrane

nature of the protein, little advancement had been made in their characterization and in identifying agonists and antagonists of the cotransporter.

The present invention is based in part on cloning of nucleic acid molecules from salamanders, rats and humans that encode proteins that are 1:3 cotransporters of sodium and bicarbonate, hereinafter the NBC proteins or NBC. The cloning of nucleic acid molecules that encode members of the NBC family of proteins makes possible 1) the isolation and characterization of other NBC- and/or BT-encoding nucleic acid molecules, 2) the characterization of members of the NBC family of proteins, 3) the ability to screen for agonists and antagonist of the NBC protein, 4) the diagnosis of biological and pathological processes that involve normal or aberrant NBC activity, 5) therapeutic methods of controlling/altering NBC activity/expression, 6) the identification of binding partners of the NBC proteins, and 7) generation of animal models in which NBC expression has been altered. The NBC proteins can be used as agents, or serve as targets for agents, that can be used to inhibit or stimulate the activity of the sodium/bicarbonate transporter, for example, to alter extra- or intracellular pH.

## II. Specific Embodiments

### A. NBC Protein

Prior to the present invention, the existence of several families of sodium/bicarbonate transporters were know. However, because of the nature of these proteins, the purification and characterization of members of the Sodium Bicarbonate Cotransporter subfamily (NBC) and the Bicarbonate Transporter superfamily (BT) of proteins has been difficult. Using the cloned nucleic acid molecules, the present invention provides the ability to produce and isolate NBC proteins, as well as allelic variants of the NBC proteins, and conservative amino acid substitutions of the NBC proteins.

As used herein, an NBC protein (or NBC) refers to a protein that has the amino acid sequence of human, rat or salamander NBC depicted in Figure 5. hNBC will refer specifically to human NBC protein, rNBC will refer specifically to rat NBC protein (~1035 amino acids in length) and sNBC will refer specifically to salamander NBC protein (~1025 amino acids in length). For the sake of convenience, hNBC, sNBC and rNBC will be collectively referred to as NBC, the NBC proteins, or the NBC proteins of the present

invention.

The term "NBC proteins" also includes naturally occurring allelic variants of the NBC proteins of the present invention, naturally occurring proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the requisite ability to cotransport 1:3 sodium and bicarbonate unless the specific allelic variant encodes an NBC protein with altered activity.

As used herein, the NBC family of proteins also includes NBC proteins that have been isolated from organisms in addition to humans, rats and salamanders. The methods used to identify and isolate other members of the NBC family of proteins are described below.

NBC is a member of the Bicarbonate Transporter (BT) superfamily of proteins. The superfamily of proteins includes the previously cloned Cl-$HCO_3$ exchanges family (AE1-3), the presently cloned 1:3 Na/$HCO_3$ cotransporter family (NBC), the 1:2 Na/$HCO_3$ cotransporter family, the 1:1 Na/$HCO_3$ cotransporter family, the K/$HCO_3$ cotransporter family and the Na-driven Cl-$HCO_3$ exchanger. However, NBC is the first member of the cotransporter (1:3 or 1:2) or cation coupled transporter/exchanger families of BT superfamily that has been cloned. Despite having partial sequence conservation with AE1-3 (chloride/bicarbonate transporter, ~30% identity over 900+ amino acids), there are important structural differences that are unique to members of the NBC family of proteins. First, the NBC amino-acid sequence has little similarity to the amino-acid sequences of the AE family at either the amino or carboxy termini. Second, NBC has a much longer 5,6 exofacial loop than the AE family. Third, NBC has a highly unusual, hydrophilic sequence ("EKDKKKKEDEKKKKKKK") near its carboxy terminus, a stretch of 17 amino acids in which each is either positively or negatively charged. In addition to these structural features, NBC differs functionally from the AE family in two important ways. First, NBC is a cotransporter (it moves $Na^+$ and $HCO^-_3$ in the same direction across the cell membrane), whereas the AE proteins are exchangers (they move $Cl^-$ and $HCO^-_3$ in opposite directions). Second, NBC is electrogenic (moving electrical charge across the membrane, and thereby changing and being influenced by cell membrane potential), whereas the AE family is electroneutral. Comparing the differences between NBC and the AE sequences will identify

portions of the NBC and AE structures that are responsible for their distinctive functional properties. These differences could then be exploited for the design of drugs, antibodies or other molecular tools.

The NBC proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the NBC protein from cellular constituents that are normally associated with the NBC protein. A skilled artisan can readily employ standard purification methods to obtain an isolated NBC protein. The nature and degree of isolation will depend on the intended use.

The NBC proteins of the present invention further include conservative variants of the NBC proteins herein described. As used herein, a conservative variant refers to alterations in the amino acid sequence that do not adversely affect the ability of the NBC protein to function as a $Na/HCO_3$ cotransporter. A substitution, insertion or deletion is said to adversely affect the NBC protein when the altered sequence prevents the NBC protein from cotransporting $Na/HCO_3$. For example, the overall charge, structure or hydrophobic/hydrophilic properties of NBC can be altered without adversely affecting the activity of NBC. Accordingly, the amino acid sequence of NBC can be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the activity of NBC.

The allelic variants, the conservative substitution variants and the members of the NBC family of proteins, will have the ability to cotransport $Na/HCO_3$. Such proteins will ordinarily have an amino acid sequence having at least about 75% amino acid sequence identity with the human NBC sequence, more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and including any conservative substitutions as being homologous. N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

The cloning of NBC-encoding nucleic acid molecules makes it possible to generate

defined fragments of the NBC proteins of the present invention. As discussed below, fragments of the NBC proteins of the present invention are particularly useful in generating domain-specific antibodies, in identifying agents that bind to an NBC protein and in identifying NBC-binding partners. Fragments of the NBC protein can be generated using standard peptide synthesis technology and the amino-acid sequences disclosed herein. Alternatively, recombinant methods can be used to generate nucleic acid molecules that encode fragments of the NBC protein. Figure 5d identifies domains that constitute preferred fragments of the NBC proteins of the present invention.

Thus, the NBC proteins of the present invention include molecules having the amino acid sequences disclosed in Figure 5; fragments thereof having a consecutive sequence of at least about 3, 5, 10 or 15 amino-acid residues of the NBC protein; amino acid sequence variants of such sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed NBC sequence; amino acid sequence variants of the disclosed NBC sequence, or their fragments as defined above, that have been substituted by another residue. Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding NBC proteins of other animal species, including but not limited to rabbit, rat, murine, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the NBC family of proteins; and derivatives wherein the NBC protein has been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope).

As described below, members of the NBC family of proteins can be used for, but are not limited to, 1) a target to identify agents that block or stimulate NBC mediated pH regulation, 2) a target or bait to identify and isolate binding partners that bind NBC, 3) methods to identify agents that block or stimulate the activity of an NBC protein and 4) a target to assay for NBC-mediated activity.

### B. Chimeras of NBC proteins

Because NBC proteins have hydropathy profiles similar to AE proteins, chimeras of NBC and AE proteins can be used as tools in defining domain structure/activity of the NBC

family of proteins. Accordingly, the present invention provides protein chimeras containing a fragment of an NBC protein of the present invention.

As used herein, a chimera, or chimeric protein, is a protein that is made up of segments that are not normally associated with each other. For example, a chimera of an NBC protein can be made up of an NBC protein in which the cytoplasmic C-terminal region is replaced by the C-terminal region of an AE protein. The preferred chimeras will contain one or more intracellular or extracellular domains of an NBC protein.

In general recombinant DNA methods are used to generate a nucleic acid molecule that encodes the chimera. The methods for generating rDNA molecules are described in detail below. A skilled artisan can readily use such procedures to generate chimeric proteins containing one or more fragments of the NBC proteins of the present invention.

### C. Anti-NBC Antibodies

The present invention further provides antibodies that selectively bind one or more of the NBC proteins of the present invention. Anti-NBC antibodies that are particularly contemplated include monoclonal and polyclonal antibodies as well as fragments containing the antigen binding domain and/or one or more complement determining regions.

Antibodies are generally prepared by immunizing a suitable mammalian host using an NBC protein, or fragment, in isolated or immunoconjugated form (Harlow, Antibodies, Cold Spring Harbor Press, NY (1989)). Regions of the NBC proteins herein described that are predicted as being highly antigenic can be readily identified using art known protein modeling methods (for example regions identified in Figure 5B. Figure 5C provides a predicted structure of an NBC protein and identifies extra and intracellular domains. Figure 5A identifies regions that are conserved amongst hNBC, sNBC and rNBC. In the examples, antibodies are described that were generated against fusion proteins consisting of residues 338-391 or 928-1035 of an NBC protein.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective.

Administration of the NBC immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the NBC protein or peptide fragment. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the transporter can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin.

As described below, anti-NBC antibodies are useful as modulators of NBC activity, are useful in immunoassays for detecting NBC expression/activity and for purifying an NBC protein.

### D. NBC-Encoding Nucleic Acid Molecules

As described above, the present invention is based in part on isolating nucleic acid molecules from salamander, rats and humans that encode members of the NBC family of proteins. Accordingly, the present invention further provides nucleic acid molecules that

encode an NBC protein as herein defined, preferably in isolated form. For convenience, all NBC encoding nucleic acid molecules will be referred to as NBC encoding nucleic acid molecules, *NBC* genes, or *NBC*. The specifically identified NBC encoding nucleic acid molecules are provided in Figures 5, 9 and 10. Sequences are provided for nucleic acid molecules that encode hNBC (human), rNBC (rat, two isoforms) or sNBC (salamander), the *hNBC*, *rNBC* and *sNBC* genes respectively.

As used herein, a "nucleic acid molecule" is defined as RNA or DNA that encodes a peptide as defined above, or is complementary to a nucleic acid sequence encoding such peptides, or hybridizes to such a nucleic acid and remains stably bound to it under appropriate stringent conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acid, however, is defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to a nucleic acid encoding an NBC protein according to the present invention.

As used herein, "stringent conditions" are conditions in which hybridization yields a clear and detectable sequence. Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium titrate/0.1% SDS at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 Tg/ml), 0.1% SDS, and 10% dintran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid

molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated NBC encoding nucleic acid molecule.

The present invention further provides fragments of the NBC encoding nucleic acid molecules of the present invention. As used herein, a fragment of an NBC-encoding nucleic acid molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the NBC protein, such as the intracellular C-terminal or N-terminal fragments, the fragment will need to be large enough to encode the functional region(s) of the NBC protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming. Fragments of the *NBC* gene that are particularly useful as selective hybridization probes or PCR primers can be readily identified using art know PCR primer selection methods. Such fragments contain regions that are conserved among *hNBC*, *rNBC* and *sNBC* and regions of homology that are shared with the previously identified AE class of proteins. Figure 5B and D identifies potential domains of the NBC proteins.

Fragments of the NBC encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding NBC proteins can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, *J Am Chem Soc* (1981) 103:3185-3191 or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the *NBC* gene, followed by ligation of oligonucleotides to build the complete modified *NBC* gene.

The NBC-encoding nucleic acid molecules of the present invention further include allelic variants of the *NBC* genes herein disclosed as well as alternative splice forms that are active in tissues other than the kidney. Specifically, the NBC-encoding nucleic acid molecules herein described are expressed in kidney cells. Identical and other forms of the NBC proteins are expressed in tissues such as the brain and the heart. The present invention contemplates such nucleic acid molecules as being within the scope of the invention. A skilled artisan can readily use the methods described below to isolate other forms of the NBC-encoding nucleic

acid molecules of the present invention.

The NBC-encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. As described above, such probes can be used to identify other members of the NBC family of proteins and as described below, such probes can be used to detect NBC expression. A variety of such labels are known in the art and can readily be employed with the NBC-encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides, biotin, chromaphores and the like. A skilled artisan can employ any of the art known labels to obtain a labeled NBC encoding nucleic acid molecule.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino-acid sequence encoded by DNA falling within the contemplated scope of the present invention.

**E. Isolation of Other NBC-Encoding Nucleic Acid Molecules**

As described above, the identification of the salamander, rat and human NBC encoding nucleic acid molecules allows a skilled artisan to isolate nucleic acid molecules that encode other members of the NBC family of proteins, as well as members of the BT superfamily of proteins, in addition to the sequence herein described.

Essentially, a skilled artisan can readily use the amino acid sequence of NBC to generate antibody probes to screen expression libraries prepared from cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified NBC protein or a fusion protein generated by recombinant techniques (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as lambda gtll library, to obtain the appropriate coding sequence for NBC, or other members of the NBC family of proteins. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme. Figures 5 B and D identify important antigenic and/or putative operative domains found in the NBC protein sequence. Such regions are preferred sources of antigenic

portions of the NBC protein for the production of probe, diagnostic, and therapeutic antibodies.

Alternatively, a portion of the NBC encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the NBC family of proteins from any mammalian organisms that contains such a protein. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives. This method was employed to isolate the hNBC- and the rNBC-encoding sequences, using the sNBC encoding sequences as a probe.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an NBC-encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other NBC encoding nucleic acid molecules. Conserved NBC sequences found in Figure 9 are regions of the *NBC* gene that are particularly well suited for use as a probe or as primers. As described above, the NBC encoding nucleic acid molecules of the present invention are particularly well suited for isolating NBC encoding nucleic acid molecules that are expressed in organs such as the brain and heart as well as nucleic acid molecules that encode the related 1:2 $Na/HCO_3$ cotransporter family, the 1:1 $Na/HCO_3$ cotransporter family, the $K/HCO_3$ cotransporter family and the Na-driven Cl-$HCO_3$ exchanger family.

### F. rDNA Molecules Containing an NBC Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain an NBC encoding sequence, or a fragment thereof. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, *Molecular Cloning* (1989). In the preferred rDNA molecules, an NBC encoding DNA sequence is operably linked to one or more expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the NBC

encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the NBC encoding sequences included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators and other regulatory elements. Preferably, an inducible promoter that is readily controlled, such as being responsive to a nutrient in the host cell's medium, is used.

In one embodiment, the vector containing an NBC encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule intrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the NBC encoding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, NJ.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form rDNA molecules that contain an NBC encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient

restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J Mol Anal Genet* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the presence of the appropriate drug for the selectable marker.

### G. Host Cells Containing an Exogenously Supplied NBC Encoding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes an NBC protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of an NBC protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the *NBC* gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line, the most preferred being cells that do not naturally express an NBC protein.

Any prokaryotic host can be used to express an NBC-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to

transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

**H. Production of an NBC Protein Using a rDNA Molecule**

The present invention further provides methods for producing an NBC protein that uses one of the NBC encoding nucleic acid molecules herein described. In general terms, the production of a recombinant form of an NBC protein typically involves the following steps.

First, a nucleic acid molecule is obtained that encodes an NBC protein, such as the nucleic acid molecule depicted in Figure 5. If the NBC encoding sequence is uninterrupted by introns, as are the sequences provided in Figure 5 and 9, it is directly suitable for expression in any host. If not, then a spliced form of the NBC encoding nucleic acid molecule can be generated and used or the intron-containing nucleic acid molecule can be used in a compatible eukaryotic expression system.

The NBC-encoding nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the NBC encoding sequences. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the NBC protein. Optionally the NBC protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is

accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with NBC encoding sequences to produce an NBC protein. Particularly well suited are expression system that result in the production of lipid vesicles containing the expressed protein. Such lipid containing vesicles are well suited for identifying agonists and antagonists of the NBC protein.

## I. $Na/HCO_3$ Transport

As provided above, NBC is a member of the BT superfamily of proteins and is involved in the cotransport of sodium and bicarbonate. This cotransport is used by cells and tissues as a means of altering extra- or intracellular pH. Accordingly, NBC can be used in methods to alter the extra- or intracellular pH or concentration of $Na^+$ or $HCO^-_3$. In general, cellular pH can be altered by altering the expression of an NBC protein or the activity of an NBC protein.

The are a number of situations in which it is desirable to alter extra- or intracellular pH or concentrations of $Na^+$ or $HCO^-_3$. Abnormal extra- or intracellular pH (as occurs in acute and chronic respirator and metabolic acidoses and alkaloses) leads to water retention, increased blood pressure, inflammation, cell proliferation (as in cancer), sperm activation/inactivation, hydroencephaly, epilepsy, altered breathing rate, glaucoma, colitis, etc. For example, acidification around the neurons in the brain reduces their electrical excitability. The acidification of the epididymis (a tissue in the testis) keeps sperm cells immobile; as the sperm pass out of the body, they are alkalinized, rendering them active.

Hence, an NBC protein or *NBC* gene expression can be used as a target for means to alter extra- or intracellular pH, or sodium or bicarbonate concentrations. For example, an *NBC* gene can be introduced and expressed in cells to increase NBC expression. This provides a means and methods for altering extra- and intracellular pH.

There are pathological conditions characterized by inappropriate extra- or intracellular pH. For example, water retention, increase blood pressure, chronic respiratory

and metabolic acidosis, inflammation, cell proliferation (as in cancer), sperm activation/inactivation, hydroencephaly, epilepsy, altered breathing rate, glaucoma, colitis, are all associated with abnormal intracellular and/or extracellular pH. Accordingly, NBC activity/expression is targeted as a means of treating these conditions. Various methods for regulating NBC activity/expression are discussed in detail below.

**J. Identification of Agents that Bind to an NBC Protein**

Another embodiment of the present invention provides methods for identifying agents that are agonists or antagonists of the NBC proteins herein described. Specifically, agonist and antagonist of an NBC protein can be first identified by the ability of the agent to bind to an NBC protein. Agents that bind to an NBC protein can then be tested for the ability to stimulate or block $Na/HCO_3$ cotransport in an *NBC*-expressing cell.

In detail, an NBC protein is mixed with an agent. After mixing under conditions that allow association of NBC with the agent, the mixture is analyzed to determine if the agent bound the NBC protein. Agonists and antagonists are identified as being able to bind to an NBC protein.

The NBC protein used in the above assay can either be an isolated and fully characterized protein, can be a partially purified protein, can be a cell that has been altered to express an NBC protein or can be a fraction of a cell that has been altered to express an NBC protein. Further, the NBC protein can be the entire NBC protein or a specific fragment of the NBC protein. Particularly useful fragments include, but are not limited to the intracellular and extracellular domain and the DIDS binding domain. It will be apparent to one of ordinary skill in the art that so long as the NBC protein can be assayed for agent binding, e.g., by a shift in molecular weight or change in cellular pH, the present assay can be used.

The method used to identify whether an agent binds to an NBC protein will be based primarily of the nature of the NBC protein used. For example, a gel retardation assay can be used to determine whether an agent binds to a soluble fragment of an NBC protein whereas patch clamping, voltage clamping, pH-sensitive microprobes or pH-sensitive chromaphores can be used to determine whether an agent binds to a cell expressing an NBC protein. A skilled artisan can readily employ numerous techniques for determining whether a particular

agent binds to an NBC protein.

Once binding is demonstrated, the agent can be further tested for the ability to modulate NBC activity using a cell or oocyte expression system and an assay that detects NBC activity. For example, voltage or patch clamping, pH-sensitive microprobes or pH-sensitive chromaphores and expression in *Xenopus* oocytes or recombinant host cells can be used to determine whether an agent that binds an NBC protein can agonize or antagonize NBC activity.

As used herein, an agent is said to antagonize NBC activity when the agent reduces NBC activity. The preferred antagonist will selectively antagonize NBC, not affecting any other cellular proteins. Further, the preferred antagonist will reduce NBC activity by more than 50%, more preferably by more than 90%, most preferably eliminating all NBC activity.

As used herein, an agent is said to agonize NBC activity when the agent increases NBC activity. The preferred agonist will selectively agonize NBC, not affecting any other cellular proteins. Further, the preferred agonist will increase NBC activity by more than 50%, more preferably by more than 90%, most preferably more than doubling the level of NBC activity.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences of the NBC protein. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the NBC protein. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to a fragment of an NBC protein.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen

based on the amino acid sequence of the NBC protein.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of the NBC protein. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the NBC protein intended to be targeted by the antibodies. Critical regions include the domains identified in Figures 5B and 5D.

**K. Uses for of Agents that Bind to an NBC Protein**

As provided in the Background section, NBC is involved in regulating intracellular and extracellular pH, as well as $Na^+$ and $HCO^-_3$ concentrations. Agents that bind an NBC protein and act as an agonist or antagonist of an NBC protein can be used to modulate biological and pathologic processes associated with NBC function and activity. In detail, a biological or pathological process mediated by NBC can be modulated by administering to a subject an agent that binds to an NBC protein and acts as an agonist or antagonist of NBC activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by NBC. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by NBC refers to the wide variety of cellular events mediated by an NBC protein. Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, pathological processes mediated by NBC include water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, cell proliferation (e.g., cancer), sperm activation/inactivation, hydroencephaly, epilepsy, glaucoma and colitis. These

pathological process can be modulated using agents that reduce or increase the activity of an NBC protein.

As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, an agent is said to modulate acidosis when the agent contributes to normal blood pH.

## L Administration of Agonists and Antagonists of an NBC Protein

Agonists and antagonists of the NBC protein can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, to treat water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, cancer, sperm activation/inactivation, hydroencephaly, epilepsy, glaucoma, colitis, an agent that modulates NBC activity is administered systemically or locally to the individual being treated. As described below, there are many methods that can readily be adapted to administer such agents.

The present invention further provides compositions containing an antagonist or agonist of an NBC protein that is identified by the methods herein described. While individual needs vary, a determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 Tg/kg body wt. The preferred dosages comprise 0.1 to 10 Tg/kg body wt. The most preferred dosages comprise 0.1 to 1 Tg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty

acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dintran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

### M. Combination Therapy

The agents of the present invention that modulate NBC activity can be provided alone, or in combination with another agents that modulate a particular biological or pathological process. For example, an agent of the present invention that reduces NBC activity can be administered in combination with other agents that effect sodium/bicarbonate cotransport. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

### N. Methods to Identify NBC Binding Partners

Because of the nature of NBC proteins, it is presently unknown whether other cellular constituents, such as signaling proteins or non-protein cofactors, are associated with NBC proteins. The cloning of the NBC encoding nucleic acid molecules herein described allows for the detection of such binding partners and their isolation, if they exist. Accordingly, another embodiment of the present invention provides methods that can be used to isolate and identify binding partners of NBC. Specifically, the NBC protein, or a fragment thereof, can be used as a capture probe to identify NBC binding partners. As used herein, an NBC binding partner is a biomolecule (such as a protein, DNA or other cofactor)

that binds to NBC and mediates NBC activity.

In detail, an NBC protein, or a fragment of an NBC protein, is mixed with an extract or fraction of a cell that expresses NBC under conditions that allow the association of a binding partner with NBC. After mixing, peptides that have become associated with NBC are separated from the mixture. The binding partner that bound NBC can then be removed and further analyzed.

To identify and isolate a binding partner, the entire NBC protein can be used. Alternatively, a fragment of an NBC protein can be used. Because the C-terminal and N-terminal of the NBC proteins are large, intracellular regions, a fragment containing either of these regions is particularly well suited for isolating NBC binding partners because such regions are likely to be the contact site of a binding partner if one exists. Alternatively, the other intracellular and extracellular domains identified in Figure 5C can be used.

As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell. The preferred source of cellular extracts will be cells that naturally express NBC. Examples of such cells include, but are not limited to the kidney, brain, heart, stomach, small and large intestines, pancreas, liver, testis, prostate, sweat and salivary glands, mammary glands, choroid plexus, ciliary body, bone and blood vessels.

Once an extract of a cell is prepared, the extract is mixed with the NBC protein under conditions in which association of NBC with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of an NBC-expressing cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the NBC with the binding partner.

After mixing under appropriate conditions, NBC is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to NBC can be used to immunoprecipitate the NBC and associated binding partner. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of nonassociated cellular constituents found in the extract, the binding partner can be dissociated from the NBC protein using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

To aid in separating associated NBC/binding partner pairs from the mixed extract, the NBC protein can be immobilized on a solid support. For example, NBC can be attached to a nitrocellulose matrix or acrylic beads. Attachment of NBC to a solid support further aids in separating a peptide/binding partner pair from other constituents found in the extract.

Alternatively, an NBC-encoding nucleic acid molecule can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the NBC encoding molecules herein described.

**O. Use of NBC Binding Partners**

Once isolated, the NBC binding partners obtained using the above described methods can be used for a variety of purposes. The binding partners can be used to generate antibodies that bind to the NBC binding partner using techniques known in the art. Antibodies that bind an NBC binding partner can be used to assay NBC activity, as a therapeutic agent to modulate a biological or pathological process mediated by NBC, or to purify the binding partner. These uses are described in detail below.

The identified binding partner can be use to identify agents that reduce or block the association of NBC with an NBC binding partner. Specifically, NBC is mixed with an NBC binding partner in the presence and absence of an agent to be tested. After mixing under conditions that allow association of NBC with the NBC binding partner, the two mixtures are analyzed and compared to determine if the agent reduced or blocked the association of NBC with the NBC binding partner. Agents that block or reduce the association of NBC with the NBC binding partner will be identified as decreasing the amount of association present in the sample containing the tested agent.

As used herein, an agent is said to reduce or block NBC/NBC binding partner association when the presence of the agent decreases the extent to which or prevents the NBC binding partner from becoming associated with an NBC protein. One class of agents will reduce or block the association by binding to the NBC binding partner while another class of agents will reduce or block the association by binding to the NBC protein.

The NBC binding partner used in the above assay can either be an isolated and fully characterized protein or can be a partially characterized protein that binds to NBC or an NBC binding partner that has been identified as being present in a cellular extract. It will be

apparent to one of ordinary skill in the art that so long as the NBC binding partner has been characterized by an identifiable property, e.g., molecular weight, the present assay can be used.

Agents that block NBC/signaling partner interaction can be agonists or antagonists of NBC activity. Such agonists and antagonists can be used essentially as described above for agents that bind to an NBC protein.

**P. Methods for Identifying NBC Expression and NBC-Mediated Biological/Pathological Process**

The present invention further provides methods for identifying cells expressing active and aberrant forms of an NBC protein as well as techniques that can be applied to diagnose biological and pathological processes associated with NBC activity, the progression of such conditions, the susceptibility of such conditions to treatment and the effectiveness of treatment for such conditions. Specifically, NBC activity can be identified by determining whether the NBC protein is expressed in a cell. Departure from normal expression, or the expression of an aberrant form, can be used as a means for diagnosing pathological conditions mediated by abnormal NBC activity/expression.

A variety of immunological and molecular genetic techniques can be used to determine if an NBC protein, or an NBC encoding mRNA, is produced in a particular cell. In one example, an extract of cells is prepared. The extract is then assayed to determine whether NBC is expressed in the cell. The degree of expression provides a measurement of the degree of NBC activity. An increase of expression over normal levels indicates an over-activity of NBC.

The measurement of NBC expression can be used as a marker for a variety of purposes. For example in identifying susceptibility or the presence of NBC mediated water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, cancer, sperm activation/inactivation, hydroencephaly, epilepsy, glaucoma or colitis.

NBC expression can also be used in methods to identify agents that increase the expression of a naturally occurring NBC gene. Specifically, nucleic acid probes that detect mRNA can be used to detect cells or tissues that express an NBC protein. Such cells or

tissues can be contacted with a test agent to determine the effects of the agent on *NBC* expression. agents that activate NBC expression can be used as an agonist of NBC activity whereas agents that decrease NBC activity can be used as an antagonist of NBC activity.

**Q. Other Methods to Control NBC Expression**

The present invention further provides additional methods that can be used to control *NBC* expression in a cell. For example, the expression of an *NBC* antisense nucleic acid molecule in a cell can be used as a means of reducing NBC activity. A skilled artisan can readily employ antisense technology with the NBC encoding nucleic acid molecules of the present invention.

NBC activity can also be altered by altering the level of expression of naturally occurring *NBC* genes using agents identified by the methods described above. Agents that increase or decrease the level of *NBC* expression can be used to regulate NBC activity within cells or tissues.

**R. Animal Models and Gene Therapy**

The *NBC* gene and the NBC protein can also serve as targets for gene therapy in a variety of contexts. For example, in one application, NBC-deficient non-human animals can be generated using standard knock-out procedures to inactivate an *NBC* gene or, if such animals are non-viable, inducible NBC antisense molecules can be used to regulate NBC activity/expression. Alternatively, an animal can be altered so as to contain an NBC or antisense-NBC expression unit that directs the expression of NBC or the antisense molecule in a tissue specific fashion. In such uses, a non-human mammal, for example a mouse or a rat, is generated in which the expression of the *NBC* gene is altered by inactivation or activation. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the NBC-deficient animal, the animal that expresses NBC in a tissue specific manner, or an animal that expresses an antisense molecule can be used to 1) identify biological and pathological processes mediated by NBC, 2) identify proteins and other genes that interact with NBC, 3) identify agents that can be exogenously supplied to overcome NBC deficiency and 4) serve as an appropriate screen for identifying mutations within *NBC* that increase or decrease activity.

For example, it is possible to generate transgenic mice expressing the human minigene for NBC in a tissue specific-fashion and test the effect of over-expression of the protein in district that normally do not contain NBC. This strategy has been successfully used for other proteins, namely *bcl-2* (Veis et al. Cell 1993 75:229). Such an approach can readily be applied to the NBC protein and can be used to address the issue of a potential beneficial effect of NBC in a specific tissue area.

In another embodiment, genetic therapy can be used as a means for modulating an NBC-mediated biological or pathological processes. For example, it may be desirable to introduce into a subject being treated a genetic expression unit that encodes a modulator of NBC expression, such as an antisense encoding nucleic acid molecule or an NBC encoding nucleic acid molecule. Such a modulators can either be constitutively produced or inducible within a cell or specific target cell. This allows a continual or inducible supply of a modulator of NBC expression within a subject.

The following examples are intended to illustrate, but not to limit, aspects of the present invention.

## EXAMPLES

### Example 1. Isolation, Sequencing, and Expression of *Ambystoma* NBC

#### Materials and Methods.

**Methods for Figure 5.** Poly(A)$^+$ RNA was isolated from whole kidneys of 50 *Ambystoma*, using guanidinium isothiocynate and phenol/chloroform extraction, and an aliquot injected into *Xenopus* oocytes for expression. Remaining poly(A)$^+$ RNA was size fractionated by preparative gel electrophoresis to yield a sized RNA sample with an enriched response. Complementary DNAs were synthesized from this size-selected poly(A)$^+$ RNA (3.5 -5 kb) using SUPERSCRIPT™ plasmid system (GibcoBRL). cDNAs were directionally ligated into pSPORT 1, and the resulting plasmids used to transform *E. coli*. About 8000 clones were plated, plasmids isolated, cRNAs transcribed *in vitro*, and injected into *Xenopus* oocytes for expression. Positive pools were identified by a depolarization and $pH_i$ decrease elicited by $Na^+$ removal in the presence of $CO_2$ / $HCO^-_3$, a response unique to electrogenic $Na/HCO_3$ cotransporters (see Figure 7B). Successively smaller pools of cDNAs were screened until a single cDNA, named *NBC*, was identified

(Figure 8).

**Methods for Figure 6.** Poly(A)$^+$ RNA was isolated with Trizol® reagent (GibcoBRL) from tissues obtained from 25 *Ambystoma tigrinum*, followed by oligo-dT selection with oligo-dT cellulose (GibcoBRL) or FastTracks (Ambion). 2 μg of tissue poly(A)$^+$ RNA was electrophoresed in a denaturing (50% formaldehyde) MOPS buffer, and then blotted and UV cross-linked to a Zeta-Probe® nylon membrane (BioRad). The blot was probed with $^{32}$P-random-hexamer-primed DNA (GibcoBRL) from the open reading frame of *NBC* using ExpressHyb (Clontech).

**Methods for Figure 7.** 50 nl of water (control, A) or RNA solution (25 ng of poly(A)$^+$ RNA) was injected into *Xenopus* oocytes. Expression was first evident at day 7 after injection, and continued until day 13. Voltage electrodes, made from fiber-capillary borosilicate and filled with 3M KCl, had resistances of 1-10 MΩ. pH electrodes were pulled similarly, and silanized with *bis*-(dimethylamino)-dimethylsilane (Fluka). Their tips were filled with hydrogen ionophore 1 cocktail B (Fluka), and back filled with phosphate buffer (pH 7.0). Electrodes were connected to a high-impedance electrometer and output data acquired by computer. pH electrodes had slopes of -54 to -57 mV / pH unit between pH 6.0 and 8.0. The $CO_2/HCO^-_3$-free ND96 contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.5). In $CO_2$ / $HCO^-_3$-equilibrated solutions, 10 mM $NaHCO_3$ replaced 10 mM NaCl. Solutions contained 100 μM ouabain to block the oocyte's native Na-K pump. In 0-$Na^+$ solutions, choline replaced $Na^+$.

**Methods for Figure 8.** 10 ng (in 50 nl) of NBC-cRNA was injected into *Xenopus* oocytes; expression was first obvious on day 3 after injection and continued until at least day 13. In butyrate solutions, 10 mM butyrate replaced 10 mM $Cl^-$. Voltage-clamp electrodes (panel D) were filled with 3M KCl and had resistances of 0.5-1.0 MΩ.

Results

The expression-cloning strategy used was unique both in the assay and the source of the poly(A)$^+$ RNA. For the assay, both voltage- and ion-sensitive microelectrodes were used to monitor simultaneously $V_m$ and $pH_i$ of *Xenopus laevis* oocytes. The protocol was to equilibrate the oocyte with $CO_2/HCO^-_3$, and then remove extracellular $Na^+$. Because the cotransporter exports more $HCO^-_3$ than $Na^+$, this maneuver leads to a positive-shift in $V_m$ and a fall in $pH_i$. When oocytes were injected with poly(A)$^+$ RNA from rabbit kidney

cortex, the expected $V_m$ and $pH_i$ signals were not detected. Reasoning that an amphibian oocyte might recognize and translate amphibian RNA better, the tiger salamander (*Ambystoma tigrinum*), the preparation in which the 1:3 sodium bicarbonate cotransporter was first described (Boron, W.F. and Boulpaep, E.L. *J. Gen. Physiol.* (1983) 81, 53-94), was used as the mRNA source. Indeed, using the above assay, the expected $V_m$ and $pH_i$ signals in oocytes injected with poly(A)$^+$ RNA obtained from *Ambystoma* kidney were observed.

Screening a *Ambystoma* kidney cDNA library for electrogenic Na/$HCO_3$ cotransporter activity yielded a 4.1 kb cDNA encoding a novel 1025 amino acid protein (NBC). Comparing the deduced amino-acid sequence of NBC to proteins in GenBank reveals no major homology to other known proteins, indicating that NBC is the first member of a new family of $Na^+$-coupled $HCO^-_3$ transporters. Interestingly, however, there is a weak homology to the AEs (~25-30% amino-acid sequence identity), so that NBC and the AEs define a new superfamily of $HCO^-_3$ transporters. NBC and the AEs share a DIDS-binding motif, KL(X)K. Based on NBC's hydropathy plot (Figure 5B), which is remarkably similar to that of the AEs, it is suggested that NBC has ten membrane-spanning domains (MSDs) (Figure 5C). The large hydrophilic loop between MSDs 5 and 6 has several consensus N-glycosylation sites, and is predicted to be extracellular.

Using high-stringency northern analysis, the localization of NBC-mRNA, from 19 *Ambystoma* tissues, was examined. A 4.1 kb message is found in the kidney, as well as bladder, brain, small intestine, large intestine, and eye (Figure 6), consistent with the known roles of Na/$HCO_3$ cotransport in these tissues. A smaller transcript of ~1.8 kb appears in kidney, bladder, skeletal muscle, and heart.

When water-injected, control *Xenopus* oocytes are exposed to $CO_2/HCO^-_3$, $pH_i$ falls and the cells depolarize (Figure 7A). Subsequently removing external $Na^+$ leads to a small hyperpolarization and no $pH_i$ change. In poly(A)$^+$ RNA-injected oocytes, on the other hand, removing $Na^+$ produces a transient depolarization and acidification of the oocyte (Figure 7B), a response unique to electrogenic Na/$HCO_3$ cotransporters. When poly(A)$^+$ RNA-injected oocytes are treated with 400 μM DIDS, the oocytes behave as water-injected controls (not shown).

Expression of the pure *NBC* clone is illustrated in Figure 8. A feature of cells injected with *NBC* vs. poly(A)$^+$ RNA is the immediate hyperpolarization caused by

applying $CO_2/HCO^-_3$ (Figure 8A), due to the inward (i.e., reverse) flux of $Na^+$ and $HCO^-_3$. Removing $Na^+$ forces NBC to operate in the outward (i.e., forward) direction, as in the renal proximal tubule, depolarizing and acidifying the cell. 200 μM DIDS blocks these $V_m$ and $pH_i$ changes. Thus, NBC is $Na^+$ dependent and blocked by DIDS.

Next, NBC's $HCO^-_3$ dependence was tested. With *NBC*-expressing oocytes, removing $Na^+$ from a simple HEPES-buffered solution causes a hyperpolarization and no $pH_i$ change (not shown). In this experiment, however, $pH_i$ is much higher than in the presence of $CO_2/HCO^-_3$. Because the cotransporter is $pH_i$ sensitive (Soleimani, M., et al., *J. Biol. Chem.* (1992) 267, 18349-18355) the acidification produced by $CO_2$ with one caused by butyrate was matched. As shown in Figure 8B, removing $Na^+$ produces the characteristic depolarization when the oocyte is acidified with $CO_2$, but a hyperpolarization when the oocyte is acidified with butyrate. As is the case with lowering $[Na^+]_O$, lowering $[HCO^-_3]_O$ at a fixed $[CO_2]$, causes a large depolarization (Figure 8C), due to $Na/HCO_3$ efflux. Conversely, raising $[HCO^-_3]_O$ hyperpolarizes the cell.

Finally, the electrogenic nature of NBC in voltage-clamp experiments was evaluated. Adding $CO2/HCO^-_3$ elicits an outward current (Figure 8D), consistent with $Na/HCO_3$ influx. Now, removing $Na^+$ elicits inward currents of similar magnitude, consistent with $Na/HCO_3$ efflux.

The NBC clone was used to isolate other members of the family and super family of proteins. An isotype variant has been identified in brain (Figure 10). The brain isoform has an open reading frame that is identical to that of the renal NBC except that, near the C terminus, there is a 98 nucleotide deletion that introduces a frame shift. The deduced amino-acid sequence of the brain isoform is different from that of the renal NBC for the 46 C-terminal amino acids, and is also 15 amino acids longer than the original renal clone. Thus, the brain clone differs from the kidney clone over its last 61 amino acids. The brain has the original renal form of NBC. Only the brain, so far, has the unique isoform. Antibodies specific for each of the different isoforms can be readily generated and can be used to distinguish between the original and brain isoform.

A fusion antigen of maltose binding protein (MBP) and a fragment of NBC, residues 338-391 (GP3) or residues 928-1035 (GP5), was used to generate polyclonal antisera in guinea pigs. The antisera was used to probe western blots of crude membranes isolated

from Ambystoma tigrinum, rat and rabbit (Figure 21). The antibodies react against the following proteins: (1) apparent MW = 130 kDa in rabbit and rat kidney, and in rabbit brain. Also in rabbit kidney, we see a 50 kDa band. In salamander kidney has a band at 160-170 kDa. All of these bands are specific (they are blocked by preincubating the antisera with the antigenic NBC sequence in either the fusion protein used to immunize the animals (guinea pigs and rabbits) or with the antigenic NBC sequence in an alternate fusion protein. In addition to the above, we also see a ~95 kDa band in the following Ambystoma tissues: fallopian tube, uterus, vagina, trachea, lung of rabbit.

The disclosed *NBC* clone encodes the renal electrogenic $Na/HCO_3$ cotransporter that mediates $HCO^-_3$ efflux and presumably has a $Na^+$:$HCO^-_3$ stoichiometry of 1:3. A functionally related cotransporter, which mediates both $HCO^-_3$ influx and efflux and has a stoichiometry of 1:3, also has been identified in retinal Müller cells (Newman, E.A. and Astion, M.L. *Glia* (1991) 4, 424-428; Newman, E.A. *J Neurosci* (1996) 16, 159-168).

Other cotransporters, presumably related to NBC at the molecular level, mediate $HCO^-_3$ influx. Cotransporters with $Na^+$:$HCO^-_3$ stoichiometries of 1:2 are found in glia (Dart, C. and Vaughan-Jones, R.D. *J. Physiol.* (1992) 451, 365-385), liver (Fitz, J.G., Persico, M., and Scharschmidt, B.F. *Am. J. Physiol.* (1989) 256, G491-G500; Gleeson, D., Smith, N.D., and Boyer, J.L. *J. Clin. Invest* (1989) 84, 312-321; Weintraub, W.H. and Machen, T.E. *Am. J. Physiol.* (1989) G317-G327), pancreas (Ishiguro, H., *et al.*, *J. Physiol.* (1996) 495, 169-178) and colon (Rajendran, V.M., *et al.*, *J. Clin. Invest.* (1991) 88, 1379-1385). Cotransporters with stoichiometries of 1:1 are present in heart (Dart, C. and Vaughan-Jones, R.D. *J. Physiol.* (1992) 451, 365-385). Consequently, the present nucleic acid sequences of *SNBC*, *RNBC* and *HNBC* can be used to isolate these members of the BT superfamily of proteins.

In summary, the newly cloned *NBC*, when expressed in *Xenopus* oocytes, has the physiologic properties of the renal electrogenic NaHCO3 cotransporter: electrogenicity, $Na^+$ dependence, $HCO^-_3$ dependence, and sensitivity to DIDS. As the first cloned $Na^+$-coupled $HCO^-_3$ transporter, *NBC* allows molecular identification of other $Na/HCO_3$ cotransporters, as well as the $Na^+$-driven $Cl\text{-}HCO_3$ exchanger.

**Example 2. Isolation and Sequencing of Rat Brain NBC.**

Materials and Methods

**Library screening for rbNBC.** A size-selected, oligo(dT)-primed λZAP®II rat-brain cDNA library (λRB-L; obtained from Dr. Terry Snutch, U. British Colombia) was plated, and the nylon filter lifts were screened at high stringency (68°C, ~15 h) with a $^{32}$P-labeled, ~600-bp portion of rat-kidney NBC (bp 2221-2845 of ORF) obtained by PCR. The degenerate PCR primers used were: 5'-gCTATA/T/CCCggCTTTgCTT/CGTIACC-3' (sense) and 5'-gAgg/ATCgTgCTgggAg/AAAIAg-3' (antisense). The filters were washed with (*i*) 2x SSC/0.05% SDS (1, 5 and 15 min. washes at ~22°C, then 2 x 20 min. washes at 44°C) and (*ii*) 0.1x SSC/0.1% SDS (3 x 20 min. washes at 44°C). Washed filters were wrapped in Saran wrap and autoradiographed. Positive plaques were isolated and replated, and a secondary screening was performed as described above. Positive secondary plaques were isolated, and the corresponding pBluescript SK(-) phagemids were excised ("rescued") from the λZAP®II vector as described in the Lambda ZAP®II library instruction manual (Stratagene, La Jolla, CA). The cloned cDNA inserts within the phagemids were sequenced by the Keck Biotechnology Resource Laboratory at Yale University.

**Library screening for new NBC isoform.** A size-selected, random-primed λZAP®II rat-forebrain cDNA library (λZAPRFB; obtained from the Molecular Neurobiology Laboratory, Salk Institute, La Jolla, CA) was plated, and the nylon filter lifts were screened at high stringency (68°C, ~18 h) with a $^{32}$P-labelled, ~600-bp portion of rat-kidney NBC (bp 1027-1603 of the ORF). EcoRI and ScaI restriction enzymes were used to excise the probe out of pBluescript containing a partial-length clone of rat-brain NBC isoform. Filter washes, secondary screening, and phagemid excision were performed as described in the above Section.

Results

**Isolation of two partial-length NBC-related clones by screening a rat-brain cDNA library with a ~600-bp portion of rat-kidney NBC (rkNBC), we isolated.** Compared to the open-reading frame (ORF) of rkNBC, the ORF of each of the partial-length clones is incomplete by ~700 base-pair (bp) at the 5' end (Figure 10). One of the partial-length clones is identical to rkNBC. The other clone (rat-brain NBC, or rbNBC) is also identical to rkNBC, except for a 97-bp deletion near the 3' end, causing a frameshift that would yield a

protein with 61 novel C-terminal amino acids.

**rkNBC and rbNBC are both present in mRNA from total brain (TB) of rat.** After synthesizing cDNA by reverse transcribing TB mRNA, we amplified the cDNA using PCR techniques and primers complementary to sequences flanking the 97-bp deletion in rbNBC (sense primer: 5'-ggCgTgTTCTTgTATATgggggTggCCTCA-3'; antisense primer: 5'-gTTCCTCCAAAgATAATCgTTgTCT-3'). We observed two bands of the expected sizes (~640 and ~540 bp) when we analyzed a portion of the reaction on a 1.5% agarose gel. We subcloned and sequenced these two PCR products to confirm their identity.

**Isolation of another partial-length, NBC-related clone by screening a rat-brain cDNA library with a ~600-bp portion of rat-kidney NBC (rkNBC), we isolated.** Compared to the open-reading frame (ORF) of rkNBC, the ORF of the partial-length clone is incomplete at the 5' end by ~400 bp and incomplete at the 3' end by ~1300 bp (Figure 11). This new NBC isoform is also identical to rkNBC, except for a 27-bp deletion near the 5' end.

## Example 3. Identification of 12 Genomic DNA Clones (PACs) with Homology to Human NBC cDNA.

Material and Methods

**hgPAC (human genomic plasmid artificial chromosome).** hgPAC's were obtained from two different sources: Genome Systems (St. Louis, MO) and Dr. Richard Lifton's laboratory (HHMI and Boyer Center for Molecular Medicine, Yale Univ., School of Medicine). Both library screenings used the same PCR-generated cDNA probe. This PCR-cDNA probe (~1.6 kb) was generated by amplification of the original human kidney clone (h912b) using SSSS_f / DPGE_r. h912b was obtained by screening a human kidney cortex lgt10 library with the EcoRI-fragment of the salamander NBC clone (aNBC-*Ambystoma* NBC). This EcoRI-fragment is a 3kb fragment which encompasses most (>95%) of the aNBC open reading frame (ORF) or amino acid coding region.

**hgPAC's from Genome Systems, Inc**

256 chr 4q (also known as 15256)

257 chr 4q (also known as 15257)

258 chr 4q (also known as 15258)

478 chr 4q at centromere(also known as 15478)

479 chr 17 q; NBC-related gene, but NOT identical to NBC (also known as 15479)

**hgPAC's from Dr. Richard Lifton's laboratory**

839 chr 4q

864 chr 4q

915 chr 4q

929 chr 4q

947 chr 4q

952 chr 4q

994 probably UNRELATED gene on chr 1

**Basic method.** One microgram of the 12 hgPACs was digested in separate reactions with three restriction endonucleases: BamHI, EcoRI, and HindIII. These 36 digests were run on a 0.65% agarose/ TBE gel. The gels were then blotted onto ZetaProbe membrane (BioRad), DNA alkali denatured, and then UV-cross-linked to the membrane. These hgPAC Southern blots were probe on separate occasions with two NON-overlapping regions of human NBC clones: h912b and imfr. Blots were prehybridized with ExpressHyb (Clontech) for 30-40 min. at 65 °C, and then probed with 1 $x10^7$ cpm /mL at 65 °C for 2-3 hours. Blots were washed 3x with 2xSSC / 0.05% SDS at room temperature over 40 min. A final high stringency washes contained 0.1xSSC / 0.1% SDS and was performed at 65 °C over 30-40 min. Blots were wrapped in saran wrap and exposed to autoradiography film at -80 °C for 12-36 hours and then developed.

**Southern blots.**

**5' Probe = h912b.** As indicated above h912b is a 5' human kidney cDNA clone, which was then amplified using PCR between SSSS_f / DPGE_r. This human 5' probe was then random hexamer primed with $^{32}$P-dCTP and run over a G-50 Sephedex column to remove free nucleotides and increase the specific activity of the probe. This 5' probe at high stringency hybridized to all hgPACs except 478, 479, and 994. Even with longer exposure

time no reactivity was evident. Banding patterns for the other clones might be suggestive of more than one gene, but the data was not conclusive.

**Imfr = 3' probe.** imfr is a probe previously discussed in the earlier iteration of this application. It is obtained from a cDNA (human melanocyte origin) found in the I.M.A.G.E consortium dbEST (EST database). This clone begin with "FLDDVIPEKDKKKK..." and continues ~ 500 bp into the untranslated region of the cDNA. Thus, "imfr" does NOT overlap with h912b, though both contain regions of the NBC cDNA(s). Again the probe was generated and purified as above, and the same Southern blots hybridized at high stringency. Only 864 was recognized by this 3' probe. Thus, only hgPAC 864 contains both the 5' and 3' ends of the NBC cDNA by Southern analysis and is therefore, presumed to contain a complete NBC gene.

**FISH analysis.** FISH localization carried out on interphase chromosome squashes of human neutropils revealed that there was indeed more than one NBC gene. As indicated above, the hgPACs fall onto 3 human chromosomes: 1, 4, and 17. 994 on chr 1 does not seem to be a true NBC related gene; however, since the hgPAC has not been sequenced it is still formally possible that 994 represents a somewhat distantly related NBC gene.

479 = 15479: This hgPAC lies on 17q and appears to be a NBC related gene (see PCR analysis below).

478 = 15478: This hgPAC lies on 4q at the centromere.

all other hgPACs: All of the remaining hgPACs lie on 4q10-12. However, there are 2 groups about 5 megabases apart. This finding has been confirmed by "fiber-FISH."

**PCR analysis.** h912b and hhNBC have a unique 5' sequence. Both are different from rat kidney NBC and are different from another published human kidney NBC (Burnham et al, JBC 1997). The start of the Burnham et al clone is identical to rNBC, i.e., "MSTE..." Our human clone all begin with "MEDE...." Further in the sequence "LISPAAER...ETARWICFEE..." our sequences and the Burnham et al sequence are identical. Several primer sets amplify products from 11 of the 12 hgPACs (only suggestive amplification of 994). 256 is undetermined.

MEDE start only: 929, 952, and possibly 994—intronless.

MSTE start only: 839, 915, 947- contains at least one intron.

Both MEDE and MSTE start : 257, 258, 864, possibly 478 and 479.

From the genes which contain both MSTE and MEDE starts, MSTE appears to PRECEDE MEDE in the genomic sequence. Thus, while it cannot be inferred that clones 929, 952, and 994 (MEDE start only) represent a separate NBC gene, clones 339, 915, and 947 (MSTE start only) are most likely distinct from the NBC gene which contain both the MSTE .... Intron.... MEDE... start.

**hgPAC 479.** MEDE start has been confirmed by sequencing.

Results

**Genomic DNA Sequence information.** To identify the genomic DNA sequences for the human form(s) of NBC, Genome Systems, Inc. used a fragment of the NBC clone IMFR (Figure 9) to screen a PAC (Plasmid Artificial Chromosome) DNA library. This screening identified 5 genomic PAC clones with homology to the human NBC. Five PAC DNA's (15256, 15257, 15258, 15478 & 15479) were identified and obtained from Genome System Inc. Additionally, Dr. Richard P. Lifton (Yale University) also screened his PAC DNA library and identified 7 additional PAC clones (839, 864, 915, 929, 947, 952 and 994). Thus, 12 clones were used in total for this experiment. Restriction digest and Southern Blot analysis of these PAC DNA's were obtained. All clones except #994 hybridized $^{32}$P-labeled NBC (human Kidney NBC). The number of unique clones could not be determined. but would not be consistent with a single clone. These same blots were also probed with the 3' end of NBC. The only PAC clone that hybridized with this probe was clone 864. Therefore the only PAC clone with this 3' sequence is 864. Using FISH (Fluorescence *In Situ* Hybridization) NBC genomic DNA (PAC) was localized to chromosomes 1, 4q and 17. Therefore there are a minimum of 3 genes. Further, there are 3 hybridizations on chr 4q. PCR was used to identify and help characterize the putative gene structure of the Human NBC gene.

**Figures 12, 13 and 14.** Representation of the NBC cDNA with location of various DNA oligonucleotide primers is shown in Figure 12. Each primer is represented by a horizontal bar and is identified by the single letter amino acid code for the first 4 amino acids encoded by DNA oligonucleotide. The sequence of the PCR primers is provided in Figure 13. Using these primers to date we can determine the location of potential introns. Each primer pair will yield a DNA fragment of predicted size (from the nucleotide sequence of NBC

cDNA). The yield of a DNA product of a size greater than that of the expected value is an indication of the area of an intron. The following is a description of the areas we have identified for putative introns. The location is identified by the clone number and the forward primer and the reverse primer (Figure 12) used for the PCR reaction and the and estimation of the additional amount of nucleotide sequence present. We have analyzed some of the PAC DNA's and found that there are regions (delineated by primer pairs) which contain a considerably larger number nucleotides than the cDNA contains (see Column 4 of Figure 14). This indicates the location of putative intron-exon boundaries as well as alternative splice sequence and information. It appears that clone 15258 does not have any intron sequence. It has also been noted that we have 3 populations of PAC DNA's as indicated by the presence of a 5' sequence of MEDE only (15256. 25258, 479)(see methods) or only a MSTE (947, 478) or both MEDE and MSTE (15257). Analyses on additional clones is conducted in the same manner as reported herein.

Summary

Twelve (12) human genomic plasmid artificial chromosomes (PACs) with homology to human NBC cDNA have been identified. Analysis of these clones has resulted in the identification of a minimum of 19 regions of these PACs putative intron DNA sequence. At least three distinct genes have been localized to Chromosomes 1, 4q and 17. Analysis of the restriction enzyme and Southern blot patterns of the PAC DNA coupled with PCR analysis demonstrates that these clones have homology to human NBC DNA.

**Example 4. Isolation and Sequencing of Human Heart NBC (hhNBC).**

Materials and Methods

**Cloning hhNBC.** The open reading frame of rkNBC was cut into three pieces with BstX I, radiolabeled with a-[$^{32}$P]-dCTP by random priming, pooled and used to screen a human heart λZAP II cDNA library. The titrated plaques ($0.64 \times 10^6$) were plated and blotted on nitrocellulose filters. Hybridization was performed in 0.5 M sodium phosphate, 7%SDS, 1% Bovine Serum Albumin at 65°C overnight. The membranes were washed at 50°C in 1× SSC/0.1% SDS, and then autoradiographed. We identified true positive plaques using PCR with pBluescript vector primers adjacent to the cloning sites to amplify the cDNA insert from each positive plaque. PCR products were separated on 1.0% agarose gel, capillary transferred to the nylon, and separately hybridized with each probe used for screening

library. Positive plaques were plated for a second screening with the same probes. We isolated four positive phage clones, and excised the inserts by rescuing the plasmids. Sequencing was performed by the Keck Sequencing Center at Yale University. We analyzed the sequence using DNAsis (Hitachi Software, San Bruno, CA).

The above approach yielded nucleotide sequence data from both the 5' end (clones 15.1, and 1.3) and the 3' end (clones 4.1 and 8.1) of the putative clone. However, we were missing the middle of the putative clone. We used poly(A)$^+$ RNA from human heart and RT-PCR to obtain the missing middle portion, including the 3' end already represented by clones 4.1 and 8.1. The upstream primer sequence was 5'-CCG GAG AAG GAC CAG CTG AAG-3', corresponding to a region near the 3' end of clone 15.1, which contains the 5' fragment of hhNBC. The downstream primer sequence was 5'-ATC AGA GTA GGG AGG AAA GAG-3', corresponding to a sequence at the TAG stop codon of clones 4.1. The result of the PCR was a DNA fragment that represented the middle and 3' end of the putative clone, which we sequenced. We ligated this PCR product to clone 15.1 (the 5' end) at the Pml I site, to obtain the putative full-length clone of hhNBC. We confirmed the sequence of this full-length clone near the ligation site. To confirm that clones 15.1 and 4.1 represent, respectively, the 5' and 3' ends of the same clone, we performed a PCR between the ATG start codon of clone 15.1 and the TAG stop codon of clone 4.1. The result was a 3.2 kb PCR product, the sequence of which corresponded to the ligation product.

Results

**Cloning of hhNBC cDNA.** To clone the $Na^+/ HCO^-_3$ cotransporter expressed in cardiac-muscle cells, we performed a low-stringency screening of a human cDNA library using rNBC as a probe. From 6.4 x $10^5$ plaques, four individual positive plaques were isolated. Two of these clones contained cDNA fragments that are identical to the 3' end of a NBC expressed in human kidney (hkNBC) (the nucleotide and amino acid sequences of hkNBC are provided in Figure 26), except the amino acid position 255, where phenylalanine replace serine. Since this sequence is present in the cDNA clone obtained from λ library, it should not be a cloning artifact generated by mis-incorporation during PCR. These clones also contained the 3' UTR. The other two clones contained a partial fragment (clone 15.1 and 1.3) corresponding to the N-terminus of hkNBC. Surprisingly, the first 85 amino acids of these clones replace the first 41 amino acid residues of hkNBC, with the ATG translation

initiation codon in a perfect Kozak sequence. With nucleotide sequence from both ends of the putative human heart NBC, we performed RT-PCR to clone the middle part. To eliminate a possible artificial "chimera" clone, two sets of separate PCR were performed: 1) one set with an upstream primer corresponding to near the ATG start codon, and a downstream primer corresponding to near the 3' end of ORF; and 2) another set with an upstream primer corresponding to the 3' end of clone 15.1 (the 3' end of clone 15.1 is identical to human kidney NBC) and the same downstream primer. Sequencing of the individual PCR products revealed a cDNA identical to hkNBC, except for the aforementioned unique 5' region. Subsequent sequencing of the PCR products revealed that it is identical to the clones obtained from the human heart library. This result thus confirms that the unique 5' end of our human NBC homologue is not a cloning artifact, but a variant of NBC.

**Sequence analysis of hhNBC.** The open-reading frame of hhNBC encodes 1083 amino acids, with a predicted molecular weight of 121 kDa (Figure 15). A detailed analysis of the deduced amino acid sequence shows that the unique N-terminus of hhNBC consists of mainly charged amino acids such as aspartate, glutamate, histidine, lysine, or arginine (Figure 16). These charged residues comprise ~50% of the unique 85 amino acids in hhNBC, higher than ~22% in the corresponding N-terminal region (41 amino acid residues) of renal NBCs. The hydropathy plot shows that the most N-terminal region of hhNBC is very hydrophilic. Since hhNBC has the predicted membrane-spanning domains (MSDs) virtually identical to hkNBC and 98% identical to rat NBC homolog (rKNBC), this suggests that the hhNBC has a strong polar or charged amino terminus in the cytoplasmic domain. A possible consequence of this major divergence at the cytoplasmic N-terminal domain in NBCs is discussed herein. With the 10 putative MSDs, three potential N-linked glycosylation sites are found at amino acid position 636, 641, and 661, consistent with the glycosylation site of hkNBC. Unlike to hkNBC, however, two putative consensus PKA sites are found; one at 49 in the N-terminal and the other at 1023 in the C-terminal region, which is conserved in the hkNBC. The 3' UTR sequences is also identical to the reported one for hkNBC (data not shown).

Electrogenic renal NBC and the anion exchangers (AEs) belong to one superfamily of $HCO^-_3$ transporter. Among AEs, hhNBC is structurally closer to AE2 or AE3 than AE1. The

N-terminal region of AE2 or AE3 is larger and contains the highly charged amino acids, whereas AE1 does not.

**Example 5.** Isolation and Sequencing of Rat Aorta (raNBC).

Materials and Methods

**Cloning hhNBC.** Degenerate primers were designed by amino acid sequence comparison among rkNBC, aNBC, and AEs. The upstream primer is AARGGIKCIGGITWYCAYCTIGAY, corresponding to 2312-2336 of rkNBC sequence and the down stream primer is ICCIGWIACRTAYTGYTCRTAIAC, corresponding to 2581-2605 of rkNBC. To obtain strong degeneracy of primers, inosine base was used for not concerned position. These primers were designed to amplify fragments corresponding to the putative transmembrane domain 8 and 9 of rkNBC. PCR was performed in first three cycles with denaturation at 94°C for 30 sec, annealing at 42°C for 1 min., extension at 72°C for 2 min., and the next 20 cycles with denaturation at 94°C for 30 sec, annealing at 50°C for 1 min., extension at 72°C for 2 min. The PCR product was then diluted to 200 fold with water and the second round PCR was done with the same upstream primer used for the first PCR and the nested downstream primer. The nested primer sequence is IGAIGIIAYICCCATRTADAGRAA, corresponding to 2715-2738 of rkNBC. The PCR product were cloned into the pCRII vector (Invitrogen). The individual PCR products obtained were sequenced at the Kech Sequencing Center at Yale University.

Results

To clone the $Na^+/ HCO^-_3$ cotransporter expressed in smooth muscle cells, we performed a PCR with degenerate primers. Degenerate primers were designed near the transmembrane domain 8 and 9, which are conserved among NBCs and AEs. After obtaining the raNBC fragments, we serially performed the primer work to identify the upstream as well as downstream sequences of raNBC until we identify the putative translation start codon and the stop codon. To perform this primer work, we used gene-specific primers and upstream or downstream primers near the vector which was used for generating library. The final full-length clone (Figure 17) was obtained by PCR with gene-specific primers corresponding to the 5' end and 3' end. The open reading frame of raNBC is 3,783 bp, encoding 1261 amino acids. The raNBC reveals ~55% homology to other NBCs and 40-45% to AE2 . A detailed analysis of the deduced amino acid sequence shows that, in contrast to rkNBC, the N-

terminus of raNBC is fairly long and consists of many charged amino The hydropathy plot of raNBC is similar to that of rkNBC, with 10 membrane-spanning domains (MSDs) (data not shown). One putative consensus PKA sites is found at 227. At least three potential N-linked glycosylation site is found at amino acid position 767-770, 777-780, and 787-790.

**Example 6.** Isolation and Sequencing of NT2.

Material and Methods

**Cloning NT2.** The Genbank™ nonredundant EST data base was queried against the raNBC. Two human EST clones (clone ID: 649838, 664517) with ~70% homology to raNBC had a highest match score with the smallest probability to be a nonspecific homology. These clones were purchased from the Genome Systems. They were sequenced at the Kech Sequence Center at Yale. The EST clone 649838 contained a 2.5 kb insert fragment consisting of more than half of the 5' coding region of NT2. The second EST clone 664517 contained a ~4 kb insert fragment corresponding the more than half of the 3' coding region of NT2. Two clones were ligated at the BamHI site present in the overlapping site.

Results

During the database search to find possible human version of raNBC or raNBC-like clones, we found that two overlapping human EST clones which show a strong similarity to raNBC (~70% homology at the amino acid level). One EST clone (clone ID: 649838) contained a 2.5 kb insert fragment consisting of more than half of the 5' coding region of NT2. It has a translation start codon with the stop signal at the upstream region. The second EST clone (clone ID: 664517) contained a ~4 kb insert fragment corresponding the more than half of the 3' coding region of NT2. This clone has a translation stop codon and the 3' untranslated region. Two clones overlapped for ~1.5 kb, representing that they together encode a full-length cDNA for NT2. We therefore ligated them at the overlapping site. The open reading frame of NT2 clone is approximately 3000 kb, encoding approximately 1000 amino acids (see Figure 18 for NT2-1A and Figure 19 for NT2-2B).

The raNBC reveals ~55% homology to other NBCs and ~40% to AEs. A detailed analysis of the deduced amino acid sequence shows that, in contrast to raNBC, both amino and carboxyl terminus of raNBC is fairly short. Some regions are specially absent from NT2. The hydropathy plot of NT2 is similar to that of raNBC (data not shown). There are at

least 10 membrane-spanning domains (MSDs). Two putative consensus PKA sites are found at amino acid number 132-135. Four potential N-linked glycosylation site are found at amino acid number 562-565, 572-575, 567-580, 582-585.

**Example 7. Isolation and Sequencing of S11.**

Materials and Methods

**Cloning of S11 cDNA clone.** By the BLAST nucleotide search in the Genebank database, we found a human EST clone (EST clone ID: 1497644) which showed a 53.7% homology to the query *Ambystoma* NBC. We purchased the EST clone from the Genome Systems Inc., and sequenced the entire cDNA insert (~2 kb). It contains a partial fragment of the full-length coding region of a NBC-like clone (S11). This clone, however, does not contain the translation start or stop codon in the reading frame.

To find the 5' end of the coding region of the clone, we performed a 5' RACE-PCR with human brain polyA RNA using the 5' RACE kit (GibcoBRL). Briefly, cDNA was made from human polyA RNA with a S11 cDNA-specific primer LSSD-R (342-369) After tailing the 5' end with CTP, PCR was done with the 5' anchor primer and the downstream gene-specific primer HANS-R (289-321). The PCR product was then diluted to 20 fold with water, and used for the second PCR with the 5' AUAP primer and the downstream gene-specific primer EHVP-R (72-99). To obtain the 3' end of the coding region of S11, we performed PCR with the λ ZAP II cDNA library made from human schizophrenic patient (gift from Dr. NL Johnston, Johns Hopkins University). The upstream gene-specific primer corresponds to SQLD-F (630-660) and the downstream primer corresponds near the polycloning site in pBluescript vector. PCR was done in 30 cycles with denaturation at 94°C for 1 min., annealing at 60°C for 2 min., and extension at 68°C for 3 min. The PCR products was diluted to 40 fold with water, and used for the second nested PCR with the primer CQEM-F and the same vector primers. The resulting PCR products were subcloned into pCRII (Invitrogen) vector and sequenced at the Keck Sequence Center at Yale.

Results

**Cloning of S11 cDNA.** By database search, we found a human EST clone with 53.7% homology to the query *Ambystoma* NBC. The clone has ~52 % sequence similarity to rkNBC, and ~70% to NT2 at the protein level. By the 5' RACE-PCR strategy, we identified the ATG translation start codon, which resides far from the end of the EST clone. By the 3'

nested PCR method, we obtained a ~ 2 kb fragment. Sequencing this fragment from one end shows that it contains nucleotide sequences which are identical to the 3' end of the EST clone. Sequencing the fragment from the other end shows no reasonable open reading frames, indicating that the ~2 kb fragment should correspond to the most 3' end of S11 clone. The middle part of the fragment is sequenced using sequencing methods well known to one skilled in the art.

**Sequence Analysis of S11 cDNA.** At present, the human brain S11 clone has been sequenced so far of 2960 nucleotides long, corresponding to 986 amino acids. We have sequence information of the almost entire cording region of S11 clone, except the last ~700 base pair of the 3' end (Figure 20). The remainder of the sequence can be easily determined using art-recognized sequencing procedures as shown herein.

S11 has a 52.4% homology to *Ambistoma* kidney NBC, and ~70% homology to raNBC or NT2, at the nucleotide level.

**Example 8. Polyclonal Antibodies to rbNBC and rkNBC.**

Materials and Methods

**Generating polyclonal antibodies.** Immunogens were prepared using the maltose-binding protein (MBP) fusion protein system. A purified PCR product encoding for either the C-terminal 46 amino acids (a.a.) of rat-kidney NBC (rkNBC), or the C-terminal 61 a.a. of rat-brain NBC (rbNBC) was ligated into the expression vector pMAL-c2 at the EcoRI restriction site. The PCR primers used to obtain the C-terminal 46 a.a. of rkNBC were: 5'-CggAATTCTCTgACTgCCCATACTCAg-3' (sense) and 5'-CgTCTAgATCAgCATgATgTgTggCgTTCAAgg-3' (antisense). The PCR primers used to obtain the C-terminal 61 a.a. of rbNBC were: 5'-CggAATTCgAgAAAgATCCTCAACATTCC-3' (sense) and 5'-CgTCTAgATTACAACgTggTTTCTgTTCC-3' (antisense). *E. coli* transfected with different pMAL-c2 constructs were grown in the presence of isopropylthiogalactoside (IPTG) to induce expression of the fusion protein gene. The *E. coli* were then harvested and lysed, and the fusion proteins were absorbed to an amylose resin. The fusion proteins were eluted from the resin in the presence of an excess of maltose. Purified fusion proteins in complete Freund's adjuvant were subcutaneously injected into rabbits.

**Preparing microsomes.** Tissues were isolated from Sprague-Dawley rats, placed in ice-

cold homogenization buffer (containing 1 tablet of protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)/ 10 mls), and homogenized using a polytron. The homogenate was then centrifuged for 15 min. at ~1000 *g* (4°C) to remove cell debris and nuclei. The supernatant was again centrifuged for 45 min at ~27,000 *g* (4°C) to pellet microsomes containing plasma and organellar membranes. The pellet was resuspended in homogenization buffer, and the protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit.

**Immunoblotting.** Microsomal proteins were separated by SDS-7.5% PAGE and visualized by Coomassie-Blue staining. The proteins in the gel were transferred onto a PVDF membrane using a semi-dry blotting apparatus and a discontinuous buffer system. Proteins on the membrane were visualized by Coomassie-Blue staining. The membrane was incubated for 30 min at room temperature (RT) in a "blocking" solution (BLOTTO: phosphate-buffered saline + 5% Carnation® dry-milk powder + 0.05% Tween-20). The membrane was subsequently incubated first for 1 h at RT in BLOTTO containing the primary antibody, and then for 1 h at RT in BLOTTO containing the secondary antibody [goat αrabbit-IgG:horseradish peroxidase (HRP)]. The membrane was washed with copious amounts of virgin BLOTTO after exposure to antibody-containing BLOTTO. In a pre-absorption experiment, BLOTTO containing the primary antibody was mixed for ~30 min with 10 μg/ml fusion protein before application to the membrane. Bound HRP was detected by chemiluminescence.

Results

A polyclonal antibody generated against either the C-terminal 61 a.a. of rbNBC (αrbNBC), or the C-terminal 46 a.a. of rkNBC (αrkNBC) predominantly recognizes a ~130 kDa protein. We also demonstrated that αrbNBC and αrkNBC recognize distinct epitopes. rbNBC is predominantly expressed in brain. αrbNBC immunoprecipitates a ~130 kDa protein from total brain of rat. Immunoprecipation techniques and antibody affinity columns enable the isolation and purification of large quantities of pure NBC protein out of complex tissue homogenates.

**Example 9. Polyclonal Antibodies to NBC-3 and NBC-5 of rkNBC.**

Materials and Methods

**Generation of Polyclonal Antisera.**

**Preparation of immunogen.** *Cloning.* We used standard procedures to prepare a fusion protein ("MBP-NBC-5") of maltose binding protein ("MBP") and the C-terminal 108 amino acids (i.e., residues 928-1035) of rat kidney NBC ("rNBC", GenBank accession No. AF004017). Briefly, the respective cDNA sequence was amplified by PCR using modified primers that added an *Eco*RI restriction site on the 5′ end, and a stop codon plus an *Xba*I restriction site on the 3′ end (sense primer: 5′-Cg gAA TTC gCg ATT ATT TTT CCA gTC ATg ATC-3′; antisense primer: 5′-Cg TCT AgA TCA gCA TgA TgT gTg gCg TTC AAg g-3′). The purified PCR product was digested with *Eco*RI and *Xba*I, repurified, and ligated into the MBP expression vector pMAL-c2 (NEB, Beverly, MA) that had been linearized with *Eco*RI/*Xba*I. The resulting construct ("pMAL-NBC-5") was transfected into *E. coli* (DH5α, GIBCO BRL, Gaithersburg, MD) and propagated in Luria-Bertani medium containing 100 μg/ml ampicillin ("LB/Amp"). Bidirectional sequencing of the purified plasmid confirmed the absence of frameshifts or mutations.

In addition, we also generated a fusion protein containing amino acids (a.a.) 338-391 of rNBC ("MBP-NBC-3"). These residues correspond to a region of rNBC just N-terminal to the first putative transmembrane segment. A third fusion protein contained the α-fragment of β-galactosidase ("MBP-βgal"; transcribed from wildtype pMAL-c2). A fourth fusion protein ("MBP-NHE3") contained the C-terminal 131 amino acids of rabbit NHE3

(Biemesderfer et al., 1997).

*Large scale expression and purification. E. coli* harboring pMAL-NBC-5 were grown at 37 C in 500 ml LB/Amp. When the culture medium had reached an optical density of 0.4 - 0.6 at 550 nm, expression of the fusion protein gene was initiated by adding isopropylthiogalactoside (IPTG; final concentration 0.3 mM) and allowed to proceed for 4 h. Cells were then pelleted by centrifugation (3,000 g for 15 min at 4 C) and resuspended in 25 ml "column buffer" (in mM: 50 TRIS, 300 NaCl, 1 phenylmethylsulfonyl fluoride (PMSF), 1 Na-EDTA, pH 7.4 with HCl). The cell suspension was sonicated vigorously for 20 min on ice. The sonicate was cleared from insoluble matter by centrifugation (15,000 rpm in SS-34, 30 min at 4 C) and diluted with 4 volumes of column buffer. For batch affinity purification, 10 ml of amylose matrix (NEB) was added to this cleared lysate and mixed by end-over-end rotation overnight at 4 C. The amylose beads were washed 5 times in 50 ml of column buffer before the MBP fusion protein was specifically eluted with an excess of free maltose (10 mM in column buffer, 5 ml total). Protein concentration in the eluate was determined with the BCA kit (Pierce, Rockford, IL), and the purity of the fusion protein was assessed by SDS-polyacrylamide gel electrophoresis ("SDS-PAGE") and staining with Coomassie Brilliant Blue G250.

**Immunization with fusion protein MBP-NBC-5.** Two guinea pigs (gp) and two rabbits (rab) were subjected to the following immunization protocol: day 0: 1 ml preimmune bleed; day 1: subcutaneous (s.c.) injection of purified MBP-NBC-5 in complete Freund's adjuvant (gp: 20 μg; rab: 50 μg); day 28: boost with MBP-NBC5 in incomplete Freund's adjuvant (gp: 20 μg; rab: 50 μg); day 38: 2-ml test bleed #1; day 56: boost (as on day 28); day 66: 2-ml test bleed #2. Subsequently, animals were boosted and bled in 4 week-intervals. Antisera were stored at 4 C with 0.03% sodium azide to prevent microbial growth.

**Characterization of Antisera by Heterologous Expression.**

**Expression in HEK-293 cells.** For transient expression in HEK-293 cells, the original rNBC cDNA clone, including 5′ and 3′ untranslated regions, was cloned into the NotI site of pSV-SPORT-1 (GIBCO). Near-confluent HEK-293 grown on coverslips were transfected using the DEAE-dextran method, and grown for 48 h. Untreated cells, mock-transfected cells, and cells transfected with only the "empty" vector were used as controls. We studied expression of rNBC by indirect immunofluorescence microscopy. Briefly, cells were fixed

in 3% paraformaldehyde/PBS, permeabilized in 0.3% Triton-X-100/PBS, blocked in 20% goat serum/PBS, and incubated for 1 h at room temperature with the respective sera diluted 1:100 in 20% goat serum/PBS. After washing the sample three times in PBS, we incubated it for 1 h with a rhodamine conjugate of a goat anti-rabbit IgG antibody (Zymed Laboratories Inc., CA), diluted 1:2,000 in 20% goat serum/PBS. After three 30-min washes in PBS, the coverslips were mounted on glass slides in Aqua-mount (Learner Inc., PA) and examined on a Zeiss Axiophot fluorescence microscope. For micrographs, a 100-ASA black-and-white film (T-Max 100, Kodak) was used.

**Expression in oocytes of Xenopus laevis.** cRNAs coding for rNBC and for murine anion exchanger isoform AE-2 were transcribed from pTLN-2 or pBluescript plasmid vectors, respectively, and injected into stage V-VI oocytes of *X. laevis*. Expression of the respective ion transporters was assessed eight days later by monitoring membrane potential and intracellular pH ($pH_i$) changes in response to removal of external $Na^+$ and $Cl^-$ using microelectrodes. Subsequently, Triton-X-100 extracts of individual oocytes were prepared as described elsewhere, except that methionine was omitted from the extraction buffer. The extracted proteins were separated by SDS-PAGE and immunoblotted as described below.

**Immunoblotting of the Native Renal $Na^+/HCO_3^-$ Cotransporter.**

**Preparation of renal membrane fractions.** Whole kidneys of adult Sprague-Dawley rats, New Zealand White rabbits, and the salamander *Ambystoma tigrinum* (Charles Sullivan, TN) were removed under anesthesia (rats: 100 mg/kg pentobarbital intraperitoneally; rabbits: same dose i.v.; salamander: submersion in 0.2% tricaine methanesulfonate). The kidneys were placed in ice-cold homogenization buffer ("HB"; in mM: 250 sucrose, 20 HEPES, pH 7.4 with HCl, 100 NaCl, 2 Na-EDTA, 1 PMSF, 0.001 leupeptin, 0.001 pepstatin) and homogenized using a Polytron). The homogenate was centrifuged (15 min at 3,000 rpm, SS-34 rotor, 4 C) and the pellet (P1), containing debris and nuclei, was discarded. The supernatant (S1) was recentrifuged (45 min at 15,000 rpm, SS-34 rotor, 4 C) and the supernatant (S2) discarded. The resulting microsomal pellet (P2), containing plasma and organellar membranes, was resuspended in HB, assayed for protein content, and stored at -20 C.

**Electrophoresis and transfer.** Proteins from the crude membrane preparation were

separated by denaturing SDS-PAGE under reducing conditions (100 mM DTT or 0.5% β-mercaptoethanol) in a discontinuous system. If not indicated otherwise, 1 mm-thick 7.5% gels were used, prepared from a premixed monomer stock (T=30%, C=2.5%; Analytical Biochemicals, Natick, MA). For the stacking gels, we used a lower concentration of acrylamide together with an increased concentration of crosslinker (T=2.5%, C=25%), rendering them more rigid and less sticky than conventional stacking gels (T=3.5%, C=2.5%). For running buffers, either a Tris/glycine buffer (375 mM Tris, 0.1% [w/v] SDS, pH adjusted to 8.8 with glycine) or a Tris/borate buffer (375 Tris, 0.1 % SDS, pH adjusted to 9.1 with boric acid) was used. Following electrophoretic separation, proteins were transferred overnight at 0.5 - 1.0 $mA/cm^2$ in a semi-dry blotting apparatus (BIO-RAD) onto PVDF membranes (Immobilon-P, Millipore) using the discontinuous Tris/glycine buffer system described by the manufacturer (Millipore).

Proteins on the membranes were stained with Coomassie Blue G250, photocopied, and subjected to the immunodetection protocol. All estimates of molecular weight (MW) were obtained by comparison to unstained standards, spaced in regular 10-kDa intervals (GIBCO), and run in the same gel. Prestained MW standards (BioRad) consistently yielded lower and more scattered MW estimates.

**Immunodetection.** For immunodetection, membranes were blocked for 30 min at ~22 C in BLOTTO, which consists of 5% [w/v] "Carnation" non-fat dry milk, 0.1 % Tween-20, in PBS (in [g/l]: 8 NaCl, 1.44 $Na_2HPO_4$, 0.24 $KH_2PO_4$, 0.2 KCl, pH 7.4). Subsequently, membranes were incubated with the antisera at the indicated dilutions in BLOTTO for 1-2 h at ~22 C, or overnight at 4 C, followed by three 10 min-washes in BLOTTO. This was followed by a 1 h-incubation with the secondary antibody (horseradish-peroxidase-labeled, affinity-purified, species-specific goat anti-IgG (whole molecule) antibodies; Sigma, St. Louis, MO; diluted 1:2,000 - 1:10,000 in BLOTTO), three 10 min-washes in BLOTTO, and one 10 min-wash in PBS. Bound horseradish-peroxidase label was detected by chemiluminescence according to the manufacturer's protocol (Pierce "SuperSignal" substrate), and documented on Kodak X-OMAT AR film.

**Antibody-preabsorption experiments.** Primary antibodies in BLOTTO were preabsorbed at ~22 C for 1 h with 10 μg/ml of one of the fusion proteins: MBP-βgal, MBP-NBC-3, MBP-NHE-3, or MBP-NBC-5. This preabsorption was followed by the standard

immunodetection protocol.

**Immunoblotting of the native $Na^+/HCO_3^-$ cotransporter from rat colon, rat stomach, human pancreas, guinea pig gall bladder, kidney, stomach, and pancreas.** Immunoblotting and antibody preabsorption experiments were performed on microsome preparations from the respective tissues as described above.

**Solubilization of the native $Na^+/HCO_3^-$ cotransporter from rat kidney.** To test for the ability of various detergents to solubilize NBC from rat kidney, rat kidney microsomes were treated at room temperature with CHAPS, Tween-80, Tween-20, Triton-X-100, CTAB, Octylglucoside, Nonidet-P-40, 4% sodium dodecyl sulfate (SDS), lithium dodecylsulfate, and 300 mM aminocaproic acid. Subsequently, insoluble material was pelleted by centrifugation at 15,000 rpm for 1 hour at +4°, and aliquots of supernatant and pellet were subjected to the standard immunoblotting protocol, using rabbit anti-(MBP-NBC-5) serum at a dilution of 1:400.

**Immunolocalization of NBC in rat, rabbit and salamander kidney.**

**Tissue Preparation for Immunohistochemistry.** *Salamander*: Female specimens of the aquatic phase *Ambystoma tigrinum*, kept at 4 C, were anesthetized by submersion in 0.2% tricaine methanesulfonate. The abdomen was opened via two paramedian incisions and one transverse suprapubic incision. Following removal of the large intestine, the kidneys were exposed and both kidneys were perfused for 15 minutes via the venous portal circulation with cold, amphibian NaCl Ringer buffered with 10 mM HEPES, pH 7.5. The perfusion solution was then switched to a periodate-lysine-paraformaldehyde fixative (PLP; in mM: 8 $NaIO_4$, 60 L-lysine-HCl, 30 $Na_2HPO_4$, 4% paraformaldehyde, in phosphate buffered saline of 200 mOsm). The kidneys were then removed, post-fixed for 4-6 hours in the same fixative, washed in PBS, and stored in 0.5% paraformaldehyde in PBS at 4 C.

*Rat and rabbit:* Adult New Zealand White rabbits and Sprague-Dawley rats were anesthetized with sodium pentobarbital, and the kidneys perfusion-fixed by first inserting a cannula into the descending aorta distal to the renal arteries. The kidneys were then perfused retrograde with PBS, pH 7.4 at 37 C, to remove blood, followed by PLP fixative (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate in PBS, pH 7.4).

For cryostat sections, kidneys were cut in half on a mid-sagittal plane and post-fixed

in PLP for 4-6 hours. The fixed tissue was then cryoprotected overnight in a 30% solution of sucrose in PBS. 5 μm-cryosections were cut on a Reichert cryostat, and mounted on gelatin-coated slides. To obtain semithin (0.5 μm) cryosections, blocks of tissue (2 - 4 mm cubes) from fixed kidneys were cut sequentially from cortex, medulla, and papilla. Thus, representative tissue from all zones of the kidney was selected, and care was taken to maintain their original orientation. Tissue blocks were post-fixed in PLP for an additional 4-6 h at room temperature, cryoprotected by a 1 hour-incubation in 2.3 M sucrose in phosphate buffer (pH 7.2) with 50% polyvinylpyrrolidone, mounted on aluminum nails, and frozen in liquid nitrogen for storage. Cutting of semithin cryosections was carried out on a Reichert Ultracut E ultramicrotome fitted with an FC-4E cryoattachment, and mounted on gelatin coated slides.

**Indirect Immunofluorescence Microscopy.** Indirect immunofluorescence microscopy was performed on either 5 μm cryosections or on 0.5 μm cryosections. Briefly, tissue sections were washed sequentially in PBS, then in 50 mM $NH_4Cl$ in PBS, and in blocking buffer (1% bovine serum albumin in PBS). This was followed by a 1 hour-incubation with the primary antiserum, diluted 1:50 in 50% goat serum in PBS. After a PBS wash, sections were incubated for 1 hour with the secondary antibody (anti-guinea pig IgG, heavy and light chain, $F(ab')_2$, conjugated to fluorescein isothiocyanate; Zymed Inc., San Francisco, CA), diluted 1:100 in 50% goat serum in PBS. Subsequently, slides were washed in PBS and mounted in *Vectashield (Vector Laboratories, Burlingame, CA)*. Micrographs were taken with a Zeiss Axiophot microscope using either Tri-X (ASA 400) or TMAX (ASA 100) films.

Results

**Generation of polyclonal antisera.** Polyclonal antibodies were successfully generated to the N-terminal third (NBC-3) and the C-terminua (NBC-5) of rat-kidney NBC (rkNBC) protein.

In order to test the specificity of our antisera for immunocytochemistry and immunoblotting applications, we expressed rkNBC heterologously in either HEK-293 cells or *Xenopus* oocytes.

**Indirect immunofluorescence microscopy in HEK-293 cells expressing aNBC.** We performed indirect immunofluorescence microscopy on HEK-293 cells using the guinea-pig

anti-(MBP-NBC-5) serum. Untreated HEK-293 cells, mock-transfected cells, and cells transfected with the "empty" vector showed only weak background fluorescence. On the other hand, virtually all cells transfected with aNBC in pSV-SPORT-1 exhibited intense rhodamine fluorescence throughout the cytoplasm and plasma membrane.

**Immunoblots of oocytes expressing rkNBC.** We next performed immunoblot experiments on *Xenopus* oocytes expressing rkNBC. As controls, oocytes were injected with either water or cRNA encoding AE2. AE2 was included as a control because, among all proteins whose primary structure is known, the anion exchangers have the highest degree of amino acid sequence similarity compared to the NBCs. In several oocytes, we used electrophysiological techniques to confirm expression of AE2 or rkNBC. On several others, we performed the immunoblots using either rabbit anti-(MBP-NBC-5) serum or rabbit anti-(MBP-NBC-3) serum. However, in a single oocyte expressing AE2 and in another expressing, we sequentially performed both the electrophysiological characterization and the immunoblot.

Microelectrode measurements of membrane potential ($V_m$) and intracellular pH ($pH_i$) in oocytes injected with cRNA encoding AE2 or rNBC showed high activity of the respective transporters, indicating strong expression of functional protein. Upon exposure to 5% $CO_2$/33 mM $HCO^-_3$, pH 7.5, the oocyte injected with AE2 cRNA responded with an initial acidification, due to $CO_2$ influx, followed by a slow recovery of $pH_i$. $Cl^-$ removal from the bath solution substantially increased the rate of alkalization, whereas returning the $Cl^-$ produced an acidification. These effects were repeatable, In fact, the $pH_i$ decrease elicited by returning $Cl^-$ was more pronounced after the second zero-$Cl^-$ pulse, consistent with the stimulation of Cl-$HCO_3$ exchange at high $pH_i$ . Consistent with the presence of an electroneutral Cl-$HCO_3$ exchanger is the observation that, changing the extracellular $Cl^-$ concentration had no specific effect on $V_m$; only a nonspecific drift, presumably due to sealing-in of the electrode, is visible. Endogenous $Cl^-$ conductances have been described in X. oocytes, but did not make any detectable contribution to $V_m$ under these conditions.

The oocyte injected with rkNBC-cRNA instantly hyperpolarized by ~80 mV upon

introduction of $CO_2/HCO^-_3$, and depolarized by ~90 mV upon removal of extracellular $Na^+$. This effect of removing $Na^+$, which is the hallmark of electrogenic $Na^+/HCO^-_3$ cotransporters, was fully reversible and repeatable. Similar results have been obtained previously in oocytes expressing either salamander or rat NBC. As expected, lowering the extracellular $Na^+$ concentration reversibly lowered the rate of alkalinization. The $pH_i$ changes produced by electrogenic Na/HCO3 cotransport are relatively slow, reflecting the small surface-to-volume ratio of oocytes.

We used SDS-PAGE to separate the Triton-X-100 extracts of the two oocytes from, one expressing AE2 and one expressing rNBC. These extracts contained most of the membrane proteins but very little of the abundant interfering yolk proteins. We transferred these proteins to a PVDF membrane and probed with rabbit anti-(MBP-NBC-5). The antiserum strongly recognized a single band of ~130 kDa in the extracts from the oocyte expressing rkNBC. The predicted MW of NBC from rat kidney is 116 kDa . The antiserum did not react with any proteins from the oocytes injected with water or with AE2 cRNA. We obtained identical results on oocytes injected with either water, AE2 cRNA or rNBC cRNA but not subjected to electrophysiological measurements. In these latter experiments, the results were the same with both rabbit anti-(MBP-NBC-3) serum and rabbit anti-(MBP-NBC-5) serum.

Together, these data show that the antisera raised to the NBC fusion proteins are specific for the $Na^+/HCO_3^-$ cotransporter in both immunohistochemical and immunoblotting assays.

**Immunoblotting of the native renal $Na^+/HCO_3^-$ cotransporter.** In order to identify the NBC protein in amphibian and mammalian species, we performed immunoblotting experiments with rabbit anti-(MBP-NBC-5) serum on renal microsomes from whole kidneys of salamander, rat, and rabbit. In salamander kidney the antiserum detected a single band of ~160 kDa. This is substantially greater than the 116 kDa predicted from the cDNA sequence of aNBC. In rat kidney and rabbit kidney, the predominant bands were at a MW of ~130 kDa. In rat kidney, the antiserum also routinely detected minor bands at ~100 kDa and ~85 kDa; the band below ~80 kDa was variable. In the rabbit kidney, the antisera also routinely detected the ~100 kDa band; the lower MW bands were variable.

**Antibody-preabsorption experiments on rat kidney.** The specificity of the labeling observed in the kidneys of salamander, rat, and rabbit was tested in further immunoblotting experiments. In the control lane, probed with native rabbit anti-(MBP-NBC-5) serum, we observed a major band at ~130 kDa, and two minor bands at ~100 and ~85 kDa. In lanes labeled MBP-βgal and MBP-NBC-3, we probed, respectively, with sera previously depleted of antibodies directed against either MBP-βgal (by preincubating with an excess of MBP-β gal) or MBP-NBC-3 (by preincubating with an excess of MBP-NBC-3). Even though the antisera used in lanes 2 and 3 were thus depleted of antibodies directed against MBP per se, the banding patterns were indistinguishable from the one produced by the undepleted serum in lane 1.

In a lane labeled MBP-NBC-5, we probed with anti-(MBP-NBC-5) serum that had been depleted of antibodies directed against MBP- NBC-5 (by preincubating with an excess of MBP-NBC-5). We observed no bands whatsoever. These results demonstrate that all three bands observed in lanes 1 - 3 were due to labeling by antibodies specifically directed against the NBC-5 portion of rkNBC. There is no evidence for cross-reactivity of antibodies directed against either MBP per se, or against bacterial contaminants. Because a ~130-kDa band was also observed in *Xenopus* oocytes heterologously expressing rkNBC, it is likely that the ~130 kDa protein truly corresponds to the rat-kidney $Na+/HCO^{-}3$ cotransporter protein. The nature of the two minor bands at ~100 kDa and ~85 kDa is not clear. However, the antibody-depletion experiments demonstrate that proteins in these two bands have NBC-specific epitopes. The two bands may represent proteolytic fragments of the ~130 kDa protein, or different NBC-isoforms.

**Immunoblotting of the native $Na^+/HCO_3^-$ cotransporter from rat colon.** In the mammalian colon, $HCO_3$ transport across the mucosa plays an important role for the handling of fluid and acid-base equivalents. Both secretion and reabsorption of $HCO_3$ seem to occur, probably in specialized areas of the colonic crypts or surface epithelial cells, but the cellular mechanisms are poorly understood. The presence of an $Na^+/HCO_3^-$ cotransporter has not been showed conclusively.

Our immunoblotting experiments demonstrate that an $Na^+/HCO_3^-$ cotransporter is

expressed in rat colon. The colonic $Na^+/HCO_3^-$ cotransporter has the same apparent molecular weight as the renal form, i.e., 130 kDa. The specificity of this finding is clearly demonstrated in the antibody preabsorption experiments.

**Immunoblotting of the $Na^+/HCO_3^-$ cotransporter from human pancreas.** The human pancreas secretes a fluid that is very rich in $HCO_3$ (90-120 mM) in order to neutralize the high acid content of the chymus entering the duodenum from the stomach. The cellular mechanisms of this $HCO_3$ secretion are not well understood. The presence and cellular localization of a $Na^+/HCO_3^-$ cotransporter in the mammalian pancreas has not been shown conclusively, and no data whatsoever are available on $Na^+/HCO_3^-$ cotransporter in humans. Our immunoblotting experiments demonstrate the presence of a $Na^+/HCO_3^-$ cotransporter in human pancreas of similar size as the rat kidney NBC. The antibody preabsorption experiments show the specificity of this finding. This is the first immunochemical demonstration of an NBC protein in humans.

**Immunoblotting of the native $Na^+/HCO_3^-$ cotransporter from rat stomach.** In the mammalian stomach, $HCO_3$ secretion amounts to approx. 10% of gastric acid secretion and is on of the major protective mechanisms against luminal acid and tryptic enzymes. The cellular mechanisms for $HCO_3$ secretion in the stomach are poorly understood, and little data are available on mammalian species. Our immunoblotting experiments show that rat stomach expresses a $Na^+/HCO_3^-$ cotransporter of similar molecular weight as the renal form. The specificity of the labeling of this protein is confirmed by antibody preabsorption experiments. The antibody titering experiment demonstrated the relatively high affinity of the antiserum for this protein. However, the abundance of this gastric form of NBC is relatively low compared to kidney or brain, and a somewhat lower signal-to-noise ratio is observed.

**Immunoblotting of the native $Na^+/HCO_3^-$ cotransporter from guinea pig gall bladder, kidney, stomach, and pancreas.** The immunoblotting experiment demonstrates that pigs also express NBCs in various tissues. The gall bladder epithelium appears to express NBC at a surprisingly high abundance. As in all other species studied so far, the expression is

especially high in the kidney. In contrast to the other species, the guinea pig kidney NBC migrates as a doublet on SDS-PAGE. The molecular identity of these two bands is not known; they might be different isoforms, or glycosylated and deglycosylated forms, or proteolytic fragments. Stomach and pancreas of the guinea pig do express an NBC albeit at very low levels.

**Solubilization of the native $Na^+/HCO_3^-$ cotransporter from rat kidney.** Virtually no previous knowledge exists to date about the biochemical and biophysical properties of the NBC protein. Apart from the estimates of the apparent molecular weights, we therefore attempted to characterize the efficiency of solubilization of rat kidney NBC by several detergents. The findings demonstrate that NBC can be solubilized by strong denaturants as SDS, but also by nonionic detergents as CHAPS, Triton-X-100, octylglucoside, or Nonidet-P-40. The nonionic detergents of the Tween series failed to solubilize NBC. Aminocaproic acid, which is not a classical detergent, but has been used for membrane proteins in the context of non-denaturing PAGE, efficiently solubilized a large fraction of the microsomal NBC.

**Immunolocalization of NBC in rat, rabbit, and salamander kidney.** To determine the cellular and subcellular location of the NBC protein in the kidneys of rat, rabbit and salamander, we performed immunofluorescence staining of semi-thin (0.5 mm) sections, and immunoperoxidase staining of standard (5 mm) PLP-fixed cryosections. We used guinea-pig anti-(MBP-NBC-5) serum in both cases. For each tissue, we separately assessed the specificity of the antiserum, using procedures analogous to those described above for the immunoblots. Using preimmune serum, we observed no staining, a finding that rules out the presence of antibodies against cytoskeleton components that are sometimes spontaneously found in rabbit serum. Using immune serum that had been preabsorbed to MBP-NHE-1 (i.e., devoid of NBC-specific sequences), we observed a distinct fluorescence signal in proximal-tubule cells. Finally, preabsorbing the immune serum with MBP-NBC-5 (i.e., the fusion protein used for immunization) completely abolished the fluorescence labeling (right panels). We observed similar specificity of the antiserum in comparable immunofluorescence experiments on rabbit and salamander.

**Immunolocalization of NBC in rat and rabbit kidney.** The experiments described in the previous section established that the anti-(MBP-NBC-5) serum, in combination with the

immunofluorescence protocol, detects the $Na^+/HCO^-_3$ cotransporter specifically, and at high spatial resolution. We next used this protocol to determine the distribution of guinea-pig anti-(MBP-NBC-5) immunoreactivity along rat and rabbit nephrons, systematically examining multiple semi-thin (0.5 mm) and standard (5 mm) sections for each species. A low-powered view a 5-mm-thick coronal section of a rat kidney, stained with guinea-pig anti-(MBP-NBC-5) serum using an immunoperoxidase technique shows that the staining is confined to the superficial and midcortical regions of the cortex, and is absent from the medulla. In higher-powered views of 5- and 0.5-mm sections, the staining localizes exclusively to proximal tubules. NBC immunoreactivity is consistently absent in all other cortical structures, including the thick ascending limb, cortical collecting duct, glomerulus, vasculature and interstitium.

We observed significant heterogeneity of labeling for NBC along the proximal tubule, with staining being greater in the early portions than towards the end of this segment. S1 segments were clearly stained with the anti-(MBP-NBC-5) serum. In contrast, the S3 segments, as determined by locating the proximal tubule-thin limb of Henle transition, were never stained. Because of the difficulty in identifying the boundaries of S2 segments we were not able to accurately determine at what point along the proximal tubule the staining for NBC fell below our detection limit.

The rabbit kidney exhibited the same staining pattern as we observed for the rat kidney, using the guinea-pig anti-(MBP-NBC-5).

**Immunolocalization of NBC in salamander kidney.** We next determined the distribution of NBC in the kidney of the salamander *Ambystoma tigrinum*, again examining multiple semi-thin (0.5 mm) and standard (5 mm) sections with guinea-pig anti-(MBP-NBC-5) serum. The overview of the salamander kidney revealed intensely stained tubules in a crescent-shaped zone. In all cases, the crescent-shaped zone was separated from the lateral surface of the kidney by ~2mm of unlabeled tissue. This crescent-shaped zone overlaps the more medial glomerular zone. In other sections, the crescent-shaped zone is more lateral, separated from the glomeruli by a region of unlabelled segments. However, in no case were these intensely stained tubules seen medial to the glomeruli. By their position within the kidney, these intensely stained tubule segments can be identified as "late distal tubules", as defined by Planelles and Anagnostopoulos. These intensely stained tubules probably even

match the narrower definition of the "late distal tubule" by Yucha and Stoner.

Higher magnification shows that these cells lack a brush border and have a distinct distal-tubule morphology. In addition, staining for NBC in the salamander kidney shows the protein to be localized exclusively in the basolateral membrane. In the thicker proximal tubules, where the electrogenic $Na/HCO_3$ cotransporter was first identified, we detected only weak staining. We saw no staining in any structures other than proximal and "late distal" tubules. Thus, like the staining pattern in the mammal, the staining pattern in the salamander is strictly basolateral. However, unlike the mammal, the salamander exhibits staining predominantly in the distal tubule.

**Western Blot of Crude Membrane Extracts Isolated from Salamander, Rat and Rabbit.** Polyclonal antisera was obtained from guinea pigs immunogenized with a fusion antigen of MBP to a fragment of NBC, residues 338-391 (GP3) or residues 928-1035 (GP5), and was used to probe western blots of crude membranes isolated from Ambystoma tigrinum, rat and rabbit (Figure 21).

**Summary.** We have demonstrated the ability of the utilized antisera to detect NBC proteins from amphibian (e.g., salamander) and mammalian (e.g., rat, rabbit, guinea pig and human) species. Also, we have demonstrated the ability of the utilized antisera to detect these proteins in various tissues (e.g., kidney, brain, colon, stomach, pancreas, gall bladder and small intestine) of these animals.

**Example 10.** <u>Tissue Distribution of hhNBC mRNA.</u>

<u>Material and Methods</u>

**Northern blot.** A human multiple tissue Northern blot (catalog number 7760-1) was purchased from Clontech (Palo Alto, CA). $^{32}P$-labeled, random-hexamer-primed cDNA probe was made from the unique 5' region of the hhNBC (+7 to +271). Hybridization was performed in the ExpressHyb (Clontech) hybridization buffer at 68°C for two hours, with the probe concentration of $0.83 \times 10^6$ cpm/ml. The membranes were washed at 37°C in 2× SSC/0.1% SDS for 40 min and then at 50°C in 0.1× SSC/0.1% SDS for 1.5 hours, and then autoradiographed for 7 days.

<u>Results</u>

Using a unique 5' region of hhNBC as a probe, we performed a high-stringency northern blot among different tissues. The probe hybridized to a ~9 kb transcript. Surprisingly, the

tissue distributions of hhNBC reveal a strong expression in pancreas and low expression in heart and brain. Kidney appears to express a minimal level of hhNBC. In a previous report (Burnham et al., 1997), the same Northern blot, when the 3' region of hkNBC was used as a probe, showed strong expressions in both pancreas and kidney with the similar signal intensity. Since hhNBC and hkNBC share the same 3' region, the signal in pancreas in the previous report should correspond to hhNBC transcript. The absence of renal NBC in the pancreas is also reported in *Ambystoma* tissues. Our Northern blot analysis therefore suggests that hhNBC is pancreas-specific, even though it was cloned from heart tissue.

**Experiment 11. Tissue Distribution of raNBC mRNA.**

Material and Methods

Northern blot. The 3' region (2227-3063) of raNBC were random-primed using the Random priming kit (GibcoBRL). The multiple tissue Northern blots for rat or human were purchased from Clontech (Palo Alto, CA). Hybridization was performed in the ExpressHyb hybridization buffer (Clontech) at 68°C for one hour, with 1-2 × $10^6$ cpm/ml. The membrane was then washed at room temperature in 1X SSC/0.1% SDS for 40 min, and at 50°C in 0.1X SSC/0.1% SDS for 1.5 hours. The blot was then autoradiographed for 24 hours.

Results

The radiolabeled raNBC probe detects strong signals in rat spleen (~7.5 kb) and at moderate levels in heart and kidney. At low level, brain, lung, liver also express raNBC. In testis, raNBC detects a smaller (~4.1 kb) transcript. The ~7.5 kb transcript is also weakly present in testis.

**Experiment 12. Tissue Distribution of NT2 mRNA.**

Material and Methods

**Northern blot.** The human EST clone (clone ID: 649838), which contains a 2.5 kb fragment of NT2 was digested with NotI and XhoI. The resulting insert DNA was random-primed using the Random priming kit (GibcoBRL). The multiple tissue Northern blots for human were purchased from Clontech (Palo Alto, CA). Hybridization was performed in the ExpressHyb hybridization buffer (Clontech) at 68°C for one hour, with 1-2 × $10^6$ cpm/ml. The membrane was then washed at room temperature in 1X SSC/0.1% SDS for 40 min, and at 50°C in 0.1X SSC/0.1% SDS for 1.5 hours. The blot was then autoradiographed for 36 hours.

Results

The radiolabeled NT2 probe detects strong signals in human peripheral blood leucocytes, testis, spleen, and skeletal muscle (~9 kb). The signal is also detectable in other tissues such as colon, placenta, kidney, and heart. In contrast to raNBC, which detects a major 4.1 kb transcript in testis, NT2 detects the same 9 kb transcript. The ~5 kb transcript is also weakly present in testis.

**Experiment 13.** **Oocytes Expressing hhNBC.**

Material and Methods

**Membrane isolation and Western blot analysis.** Groups of 4 oocytes expressing hhNBC, rkNBC, or control were washed with phosphate buffered saline (PBS) and homogenized in 0.5 ml of fresh hypotonic lysis buffer (7.5 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 μg/ml pepstatinA, 1 μg/ml leupeptin). The cellular debris were removed by centrifuging at 810× *g* for 5 min. The supernatant was then centrifuged at 15, 000× *g* for 30 min at 4°C to collect the membranes. The pellets were gently washed with the lysis buffer and were dissolved in 40 μl protein loading buffer containing 2% SDS. The samples were loaded on 7.5% SDS-PAGE and transferred to PVDF membrane. Blots were pre-incubated (1 hr) in blocking buffer containing 0.2% I-block (Tropix, Bedford, MA), 0.05% Tween 20 in Tris-buffered saline (TBS; 50 mM Tris, pH 7.4; 150 mM NaCl), then incubated with an antibody specific to the C-terminus of rkNBC (1:500). After several washes with TBS containing 0.05% Tween 20, blots were incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma) (1:2000 in blocking buffer) for one hour. Blots were washed, and developed by HRP/hydrogen peroxide catalyzed oxidation of luminol under alkaline condition (Pierce, Rockford, IL).

**Oocyte experiments.** Oocytes of *Xenopus laevis* were obtained as described in Romero et al. (1997) using collagenase (type 1A; Sigma). The open reading frame of hhNBC was ligated to the EcoRI site of pGH19. Plasmids encoding hhNBC cDNAs were linearized with NotI prior to transcription, and then *in vitro* transcribed with the mMessage mMachine kit (Ambion, Austin, TX) using T7 RNA polymerase. To obtain a maximum production of a full length transcript, the ratio of cap analog to GTP was decreased by increasing the amount of GTP to 3 mM final. The defolliculated oocytes (Stage V and VI) were injected with 50 nl of RNA (0.5 mg/ml) or water, and incubated in the OR3 buffer (50% Leibovitz L-15 media with L-glutamine, 5 mM HEPES, pH 7.5) supplemented with 5 U/ml

penicillin/Streptomycin. Injected oocytes were maintained for 3-7 days at 18°C before use.

To prepare the pH electrode, a borosilicate glass capillary (1.5 mm OD, Frederick Haer Co., MD) was pulled, dried in an oven at 200°C for at least 2 h, and vapor silanized with bis(dimethylamino)-dimethyl silane (Fluka) in a closed vessel. The electrode tip was then filled with hydrogen ionophore 1 cocktail B (Fluka), and back filled with phosphate buffer (pH 7.0) (Amman, 1981) connected to a high-impedance electrometer (FD-223, WPI, Inc) and calibrated in standard solutions of pH 6 and 8. The slope was generally 55-57 mV/pH unit.

**Solutions.** The standard ND96 contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.5). The 1.5% $CO_2$ / 10 mM $HCO^-_3$-equilibrated solution was by substituting 10 mM $HCO^-_3$ for NaCl to maintain a constant ionic strength. In 0 $Na^+$ solutions, the $Na^+$ substitute was choline. In 0 $Cl^-$ solutions, the $Cl^-$ substitute was gluconate.

Results

**Immunoblot experiments on oocytes expression hhNBC.** To study the functional properties of hhNBC, we expressed hhNBC into Xenopus oocytes by injecting hhNBC cRNA. We first tested its expression by Western blot analysis with antibody against the cytoplasmic C-terminus of rkNBC. Both hhNBC and rkNBC share ~98% amino acid identity at the C-terminal region. Oocytes injected with rkNBC cRNA expressed a band with an apparent molecular mass of 130 kDa, which is expected from the deduced amino acid of rkNBC. The same antibody recognized a band in oocytes injected with hhNBC, with an apparent molecular mass of ~140 kDa. This slightly higher band is expected because of the presence of 85 amino acid residue at the N-terminus of hhNBC. Since the *in vitro* translated product of hhNBC in rabbit reticulocyte system revealed an expected 121-kDa product, the higher molecular mass in oocytes represents a glycosylated product of hhNBC. Antibody also recognized an additional immunoreactive band (~120 kDa), which appears to be one under proteolysis. Water-injected control oocytes showed no immunoreactivity.

**Functional properties of hhNBC expressed in *Xenopus* oocytes.** Oocytes expressing hhNBC were assessed to $HCO^-_3$ transport by monitoring the recovery of intracellular pH ($pH_i$) during 1.5% $CO_2$/10 mM $HCO^-_3$ exposure. Applying $CO_2/HCO^-_3$ causes a slow and sustained decrease in $pH_i$ in oocytes injected with hhNBC-cRNA. The $CO_2/HCO^-_3$ caused

rapid hyperpolarization in oocytes expressing hhNBC (n>6), whereas modest, slowly developing depolarization in water-injected oocytes. These effects are completely reversed upon removal of the $CO_2/HCO^-_3$. As $CO_2$ diffuses into the cell and produces $HCO^-_3$, and because $HCO^-_3$ is transported in by aNBC, the hyperpolarization in wanes. However, since $V_m$ is always more negative in the presence than absence of $CO_2/HCO^-_3$, the direction of electrogenic $Na^+/ HCO^-_3$ cotransport is *inward* in oocytes.

To test if the transporter is coupled to $Na^+$, we then removed $Na^+$ from the bath in the continued presence of $CO_2/HCO^-_3$. Removing extracellular $Na^+$ (by replacing with choline) elicited a rapid depolarization, as more than one $HCO^-_3$ exits the cell with each $Na^+$. In oocytes injected with water, removing $Na^+$ lead to a small hyperpolarization (reflecting the low $Na^+$ conductance of oocytes) as previously observed. Since the transporter is $Na^+$ coupled, $Na^+$ removal caused a slow $pH_i$ decrease, reflecting the efflux of $HCO^-_3$. Returning $Na^+$ to the outside of the oocyte reversed the Vm and $pH_i$ changes. To test if the transporter requires Cl, we removed Cl from the bath by replacing with gluconate. Removing extracellular Cl- in the presence of $CO_2/HCO^-_3$ did not change the rate of $pH_i$ recovery, as the transporter accelerates. The change in Vm evoked by $CO_2/HCO^-_3$ was blocked by the stilbene derivative DIDS.

**Experiment 14.** <u>**Oocytes Expressing NT2.**</u>

<u>Material and Methods</u>

**Oocyte experiments.** Oocytes of *Xenopus laevis* were obtained as described above. The open reading frame of NT2 was ligated to the XhoI/HindIII sites of pGH19. Plasmids encoding NT2 cDNA were linearized with NotI prior to transcription, and then *in vitro* transcribed with the mMessage mMachine kit (Ambion, Austin, TX) using T7 RNA polymerase. The final concentration of GTP was 3 mM, which would increase the transcription efficiency to obtain full length transcript. The defolliculated oocytes (Stage V and VI) were injected with 50 nl of RNA (0.25 mg/ml) or water, and incubated in the OR3 buffer (50% Leibovitz L-15 media with L-glutamine, 5 mM HEPES, pH 7.5) supplemented with 5 U/ml penicillin/Streptomycin. Injected oocytes were maintained for 4-6 days at 18°C before use. The pH electrode was prepared as described previously.

**Solutions.** The standard ND96 contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES (pH 7.5). The 1.5% $CO_2$ / 10 mM $HCO^-_3$-equilibrated solution

was by substituting 10 mM $HCO^-_3$ for NaCl to maintain a constant ionic strength. In 0 $Na^+$ solutions, the $Na^+$ substitute was choline.

Results

**Functional properties of NT2 expressed in *Xenopus* oocytes.** Oocytes expressing NT2 were assessed to $HCO^-_3$ transport by monitoring the recovery of intracellular pH ($pH_i$) during 1.5% $CO_2$/10 mM $HCO^-_3$ exposure. Applying $CO_2/HCO^-_3$ causes a slow and sustained decrease in $pH_i$ in oocytes injected with NT2-cRNA. The $CO_2/HCO^-_3$, however, did not hyperpolarize the membrane and instead slowly depolarized, similar to water-injected oocytes. This indicates that NT2 is electroneutral. These effects are completely reversed upon removal of the $CO_2/HCO^-_3$. As $CO_2$ diffuses into the cell and produces $HCO^-_3$, the $pH_i$ was slowly recovered because $HCO^-_3$ is transported into oocytes.

When we removed $Na^+$ from the bath in the continued presence of $CO_2/HCO^-_3$ oocytes elicited a slow hyperlarization, again as seen in oocytes injected with water. Since the transporter is $Na^+$ coupled, $Na^+$ removal caused a slow $pH_i$ decrease, reflecting the efflux of $HCO^-_3$.

**Experiment 15. $HCO^-_3$ Dependence of rkNBC and akNBC.**

Materials and Methods

**Novel assay for determining the $HCO^-_3$ dependence of rkNBC and akNBC.** We applied a new method of the "test-standard assay" in order to study the external $HCO^-_3$ dependence of the protein of Na/$HCO^-_3$ cotransporter expressed in *Xenopus* oocytes which was encoded by rkNBC cDNA.

We injected *Xenopus* oocytes with cRNA for rkNBC. We superfused the oocytes with "test" or "standard" solutions at 8 ml/min flow rate for less that 60 seconds each. We showed, that under two-electrode voltage clamp conditions, the transition from HEPES to $CO_2$/HCO3 -buffered solutions produced an immediate outward current in the membrane of the oocyte that expressed rkNBC.

By using our new method of the "test-standard assay", we increased the $HCO^-_3$ concentration from zero - in the HEPES solution to one of the "test" bicarbonate solution. We maintained a constant $pH_o$ of 7.5 by making appropriate changes in [$CO_2$]. In this

experiment, the first bicarbonate solution was "standard" solution, which contained 33 mM $HCO_3^-$. Then we applied our first "test" solution, contained 66 mM $HCO_3^-$. In a similar way, we examined the effect of other test solutions, with concentrations ranging from 0.33 to 99 mM. Each "test" pulse is bracketed by 2 standard pulses. We normalized the "test" outward currents to the bracketing outward currents at the "standard" $HCO_3^-$ concentration (see Figure 22A). The results of experiments are shown in Figure 22B. Relative delta current for rkNBC are plotted against the bicarbonate concentration. We fitted the data to a modified form of the Mechaelis-Menten equation for normalized data. An apparent $K_m$ is $9.8 \pm 0.6$ mM.

**External bicarbonate dependence of akNBC expressed in oocytes.** We applied this new method of "test-standard assay" in order to study the external $HCO_3^-$ dependence of the protein of Na/$HCO_3^-$ cotransporter expressed in *Xenopus* oocyte which was encoded by akNBC cDNA (Figure 22A).

We injected *Xenopus* oocytes with cRNA for akNBC. We used microelectrodes to measure membrane potential. We superfused the oocyte with "test" or "standard" solutions at 8 ml/min flow rate for less that 60 seconds for each solution. We showed that the fast transition from HEPES to $CO_2/HCO_3^-$ elicits an immediate negative shift of the membrane potential ($DV_m$), due to the inward electrogenic transport of at least three $HCO_3^-$ and one $Na^+$.

By using our new method of the "test-standard assay", we increased the $HCO_3^-$ concentration from zero in the HEPES solution to one of the "test" bicarbonate solution. We maintained a constant $pH_o$ of 7.5 by making appropriate changes in $[CO_2]$. In this experiments, the first bicarbonate solution was "standard" solution, which contained 33 mM $HCO_3^-$. Then we applied our first "test" solution, contained 66 mM $HCO_3^-$. In a similar way, we examined the effect of other test solutions, with concentrations ranging from 1.65 to 99 mM. Each "test" solution is bracketed by 2 standard solutions. We normalized the $DV_m$ of each "test" to the $DV_m$ of the bracket "standard" solutions.

The results of experiments are shown in Figure 23B. Relative delta voltage for

akNBC are plotted against the bicarbonate concentration. We fitted the data to a modified form of the Mechaelis-Menten equation for normalized data. An apparent $K_m$ is 12.1 ± 0.8 mM.

**Effect of high concentrations of $SO_3^=$ and SO4= on external $HCO_3^-$ dependence.** It has been proposed that the electrogenic Na/$HCO_3^-$ cotransporter has 3 separate binding sites for Na, $HCO_3^-$ and $CO_3^=$ ions; and in addition, that $SO_3^=$ divalent ions can substitute for $CO_3^=$ and transported by NBC. We used exactly the same new method of "test-standard assay" in order to study the effect of a high concentration of $SO_3^=$ or $SO_4$= on the external $HCO_3^-$ dependence of the outward currents via rkNBC expressed in *Xenopus* oocytes (Figure 24A). We used 33 mM total sulfite. To investigate the effect of divalent ions per se, we used 33mM of the sulfate.

The results of sulfate and sulfate experiments are summarized in Figure 24B. Relative delta current for akNBC are plotted against the bicarbonate concentration. We fitted the data to a modified form of the Mechaelis-Menten equation for normalized data. Neither the apparent Km nor the apparent $V_{max}$ values were affected by the presence of high concentration of $SO_3^=$ or $SO_4^=$.

The effect of $SO_3^=$ on DIDS sensitive component of $HCO_3^-$ -induced $pH_i$ increase in *Xenopus* oocytes expressing rkBC (Figure 25).

Results

We have demonstrated the utility of a new method of "test-standard assay" to characterize the electrophysiological properties of the rat kidney NBC and salamander kidney NBC expressed in *Xenopus* oocytes.

These studies demonstrated that the external bicarbonate dependence of the rat kidney NBC and salamander kidney NBC expressed in *Xenopus* oocytes are similar.

These experiments show that rkNBC does not transport significant amount of $SO_3^=$ or HSO3-.

**Experiment 15. Assay for non-electrogenic NBCs.** In identifying novel NBCs, the initial assumption is that they are electrogenic. Therefore, experiments are initially performed on Xenopus oocytes using pH- and Vm-sensitive microelectrodes. Thus, the

assay will be either: (i) the original assay used to express clone Ambystoma-kidney NBC (i.e., monitoring a bicarbonate-dependent, DIDS-sensitive depolarization and pHi decrease when sodium is removed from the bath solution), or (ii) the novel "test standard assay" described above.

In the event that an NBC expressed in oocytes is not electrogenic, one skilled in the art can still study the function of the NBC using a number of approaches. For example, experiments can be conducted on oocytes using pH-and Vm-sensitive microelectrodes, but we will focus our attention on the pHi changes. This approach is possible, however, because of the size of the oocytes (large volume to surface area), the pHi changes caused by activation of an NBC will be small (compared to the voltage changes for an NBC that is electrogenic, for example). However, this problem can be overcome by transiently, or stably transfecting a mammalian cell, and performing experiments in these small cells (small volume to surface area). In small mammalian cells, pHi changes elicited by activation of an NBC are relatively large and easy to characterize in detail. The ability to transfect HEK-293 cells with an NBC is documented herein elsewhere.

Many NBC-like clones that are identified herein or will be identified using the procedures set forth herein may transport other ions. In particular, there is good evidence in the literature for the presence of a K/HCO3 cotransporter in squid giant axons, and a Na-dependent Cl/HCO3 exchanger in many cell types. Both of these transporters are electroneutral. We hypothesize that the cDNAs encoding for these transporters share a high degree of homology to the original NBC. Therefore, for the NBC-like clones of this invention, as well as for related NBC genes and gene products one could easily perform experiments to determine whether or not they encode for the above-mentioned HCO3 transporters. One skilled in the art would perform experiments on oocytes (using pH- and Vm-sensitive microelectrodes) or transfected cells (using pH-sensitive dyes), and determine if the pHi changes elicited by activation of the expressed NBC require specific ions (e.g., potassium in the case of a K/HCO3 cotransporter) or can be inhibited by specific pharmacological agents (e.g., tetraethylammonium in the case of a K/HCO3 cotransporter).

Finally, one could also functionally characterize both electrogenic and electroneutral NBCs by performing ion flux assays in oocytes expressing NBCs. We could study Cl-transport with $^{36}Cl$, Na transport with $^{22}Na$, and K+ transport with $^{86}Rb$.

**Experiment 16. Isolation and Sequencing of Human Pancreas NBC (hpNBC).**

Materials and Methods

hPanK-NBC was generated by Reverse transcriptase-PCR (RT-PCR), followed by 30 cycles of PCR using human NBC-specific primers.

**Reverse Transcription Reaction.** Human pancreas poly(A)+ RNA was purchased from Clontech (Palo Alto, CA). 500 nanogram of human pancreatic poly(A)+ RNA was reverse transcribed using SuperScript II- RT (Gibco-BRL, Gaithersburg, MD) according to manufactures instructions. Method steps include: (a) 500 ng + oligo-dT to 12 uL, heat to 70 C for 10 min; (b) ice for > 1 min; (c) buffer, Mg++, dNTPs, and DTT to 19 uL; (d) mix; (e) incubate at 42 C for 5 min; (f) add 1.0uL SuperScript II-RT (200U); (g) mix with pipette; (h) incubate at 42 C for 55 min; (i) incubate at 70 C for 15 min to inactivate; and, (j) incubate at 4 C; and, (k) store at -20 C.

**PCR- Amplification.** One fortieth to one fiftieth of the RT cDNA was used to PCR between SSSS_F (forward) and ERHT_R (reverse primer). Method steps include: (a) 0.5 ul of RT in 50 uL rxn; (b) 25 uM of SSSS_f and ERTHSC_r; (c) 1 cycle(94 C for 2 min, 60 C for 2 min, 68 C for 2 min); (d) 30 cycles of (94 C for 20 sec, 60 C for 30 sec, 68 C for 3.5 min); (e) 1 cycle(68 C for 15 min, 4 C for 24 hr); and, (f) store at -20 C.

**Subcloning by TA cloning (Invitrogen) into pCR2.1.** Human pancreas RT-cDNA was amplified by PCR using SSSS_f and ERHTSC_r primer and the TaKaRa PCR kit (PanVera, Milwalkee, WI). PCR product was subcloned into pCR2.1 using the TA cloning kit from Invitrogen. The product obtained was then sequenced.

Results

The nucleotide sequence and amino acid sequence of hpNBC is provided in Figure 27.

The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom as modifications will be obvious to those skilled in the art.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in

general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

WHAT IS CLAIMED:

1. An isolated nucleic acid molecule that encodes a sodium bicarbonate transporter having an amino acid sequence selected from the group consisting of the amino acid sequence of human NBC, rat kidney NBC, rat brain isoform NBC, salamander NBC and allelic variants thereof.

2. The isolated nucleic acid molecule of claim 1, wherein said nucleic acid molecule is operably linked to one or more expression control elements.

3. The isolated nucleic acid molecule of claim 1, wherein said nucleic acid molecule is included in a vector.

4. An isolated nucleic acid molecule that encodes a member of the NBC family of proteins, wherein said nucleic acid molecule hybridizes to a nucleic acid molecule of claim 1 under conditions of sufficient stringency to produce a clear signal.

5. A host transformed to contain the nucleic acid molecule of claim 1.

6. The host of claim 5, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.

7. A method for producing an NBC protein comprising the step of culturing a host transformed with the nucleic acid molecule of claim 2 under conditions in which the NBC protein is expressed.

8. The method of claim 7, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.

9. An isolated protein comprising the amino acid sequence depicted in Figure 15, allelic variants thereof and fragments thereof that retains the ability to cotransport

sodium and bicarbonate.

10. An isolated antibody that binds to the protein of claim 9.

11. The antibody of claim 10 wherein said antibody is a monoclonal and polyclonal antibody.

12. A method for reducing the severity of a pathological state mediated by NBC comprising the step of reducing NBC expression or activity.

13. The method of claim 12 wherein said NBC expression is reduced using an RNA molecule that is complementary to an NBC encoding mRNA molecule.

14. The method of claim 12, wherein said NBC activity is reduced by contacting said NBC protein with an agent that binds to an NBC protein.

15. The method of claim 14 wherein said agent is an antibody that binds to an NBC protein.

16. A method for reducing the severity of a pathological state mediated by NBC comprising the step of increasing NBC expression or activity.

17. The method of claim 16 wherein said NBC expression is increased a nucleic acid molecule that encodes an NBC protein.

18. The method of claim 16, wherein said NBC activity is increased by contacting said NBC protein with an agent that binds to an NBC protein.

19. The method of claim 18 wherein said agent is an antibody that binds to an NBC protein.

20. A method for identifying agents that bind to an NBC protein comprising the steps of:

a) incubating a cell altered to express NBC, or a fragment thereof, with an agent to be tested, and

b) determining whether said agent binds to said NBC.

21. The method of claim 20, wherein said NBC, or NBC fragment is isolated.

22. A method to assay for NBC comprising the step of determining whether an NBC protein is expressed.

23. The method of claim 22 further comprising the steps of;

a) preparing an extract of a cell, and

b) examining the proteins of said cell extract to determine the presence of an NBC protein.

24. The method of claim 22 further comprising the steps of;

a) preparing an extract of a cell, and

b) examining the mRNA of said cell extract to determine the presence of an NBC encoding mRNA.

FIG. 1

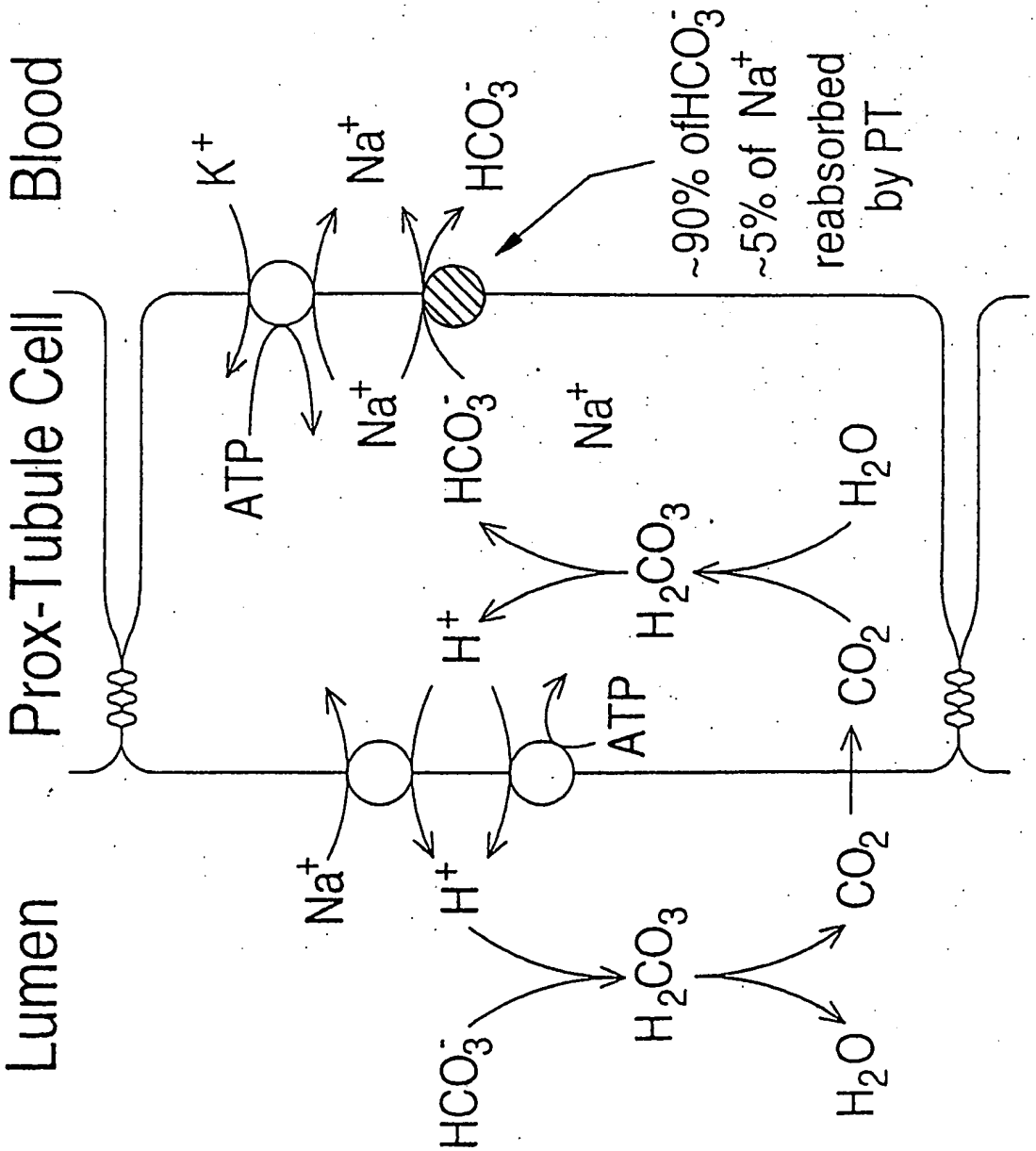

Blood
Prox-Tubule Cell
Lumen
K+
Na+
HCO3-
~90% ofHCO3-
~5% of Na+
reabsorbed
by PT
ATP
Na+
HCO3-
Na+
H2O
H2CO3
H+
CO2
ATP
Na+
H+
CO2
HCO3-
H2CO3
H2O

FIG. 2

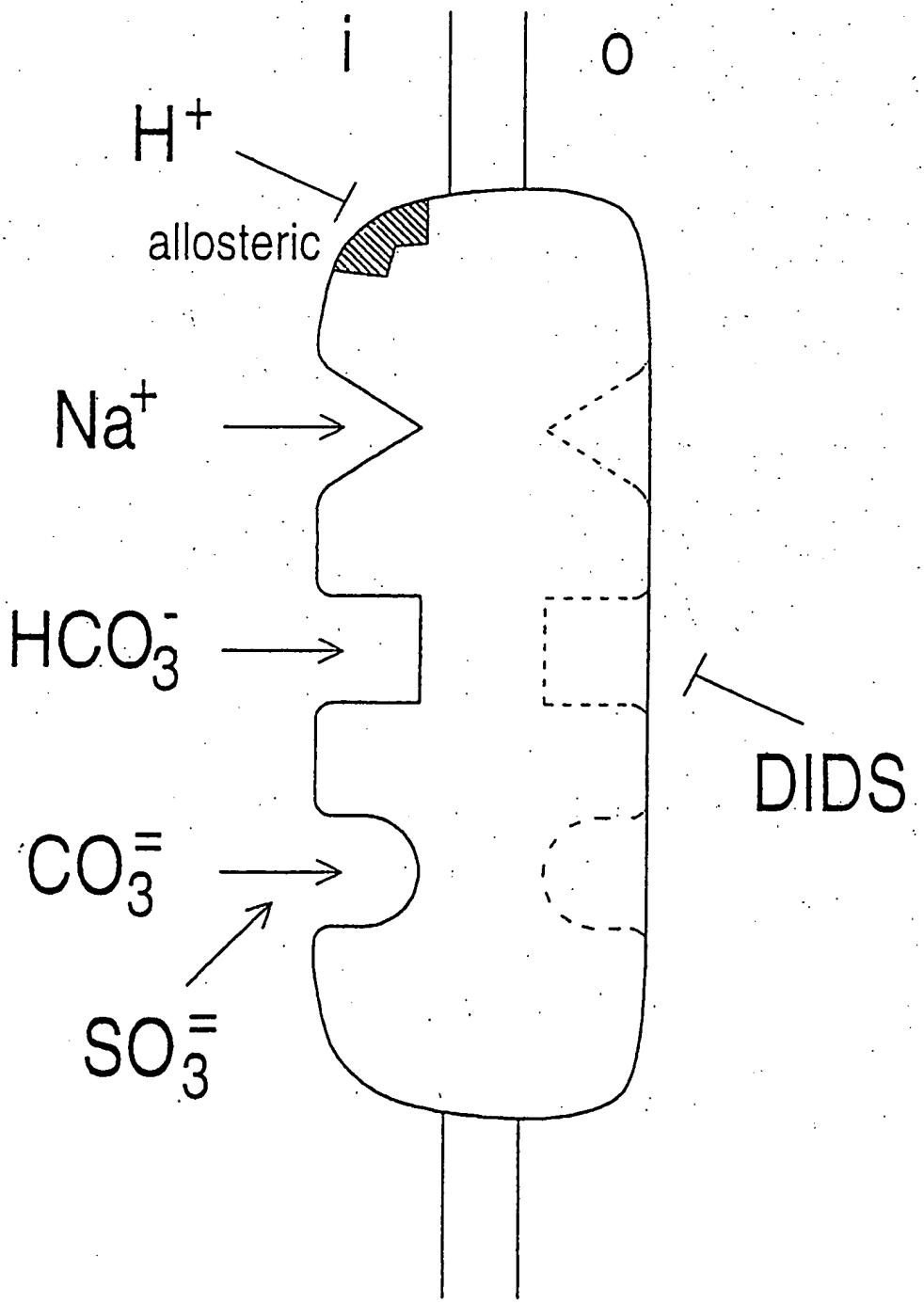
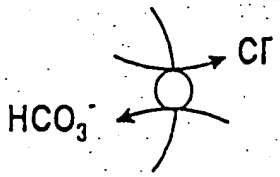
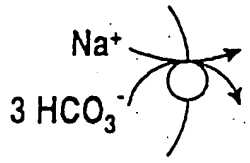
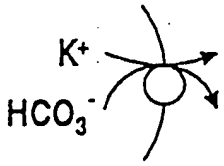
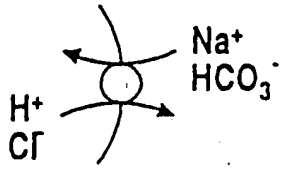
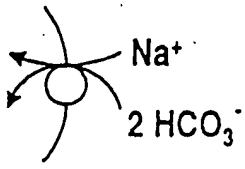
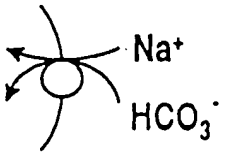


FIG. 3

| | Name | $\Delta pH_i$ | Process |
|---|---|---|---|
| *i* | Cl-$HCO_3$ exchanger | ↓ |   |
| *ii* | 1:3 Na/$HCO_3$ Cotransporter | ↓ |   |
| *iii* | K/$HCO_3$ Cotransporter | ↓ |   |
| *iv* | Na-driven Cl-$HCO_3$ exchanger | ↑ |   |
| *v* | 1:2 Na/$HCO_3$ Cotransporter | ↑ |   |
| *vi* | 1:1 Na/$HCO_3$ Cotransporter | ↑ |   |

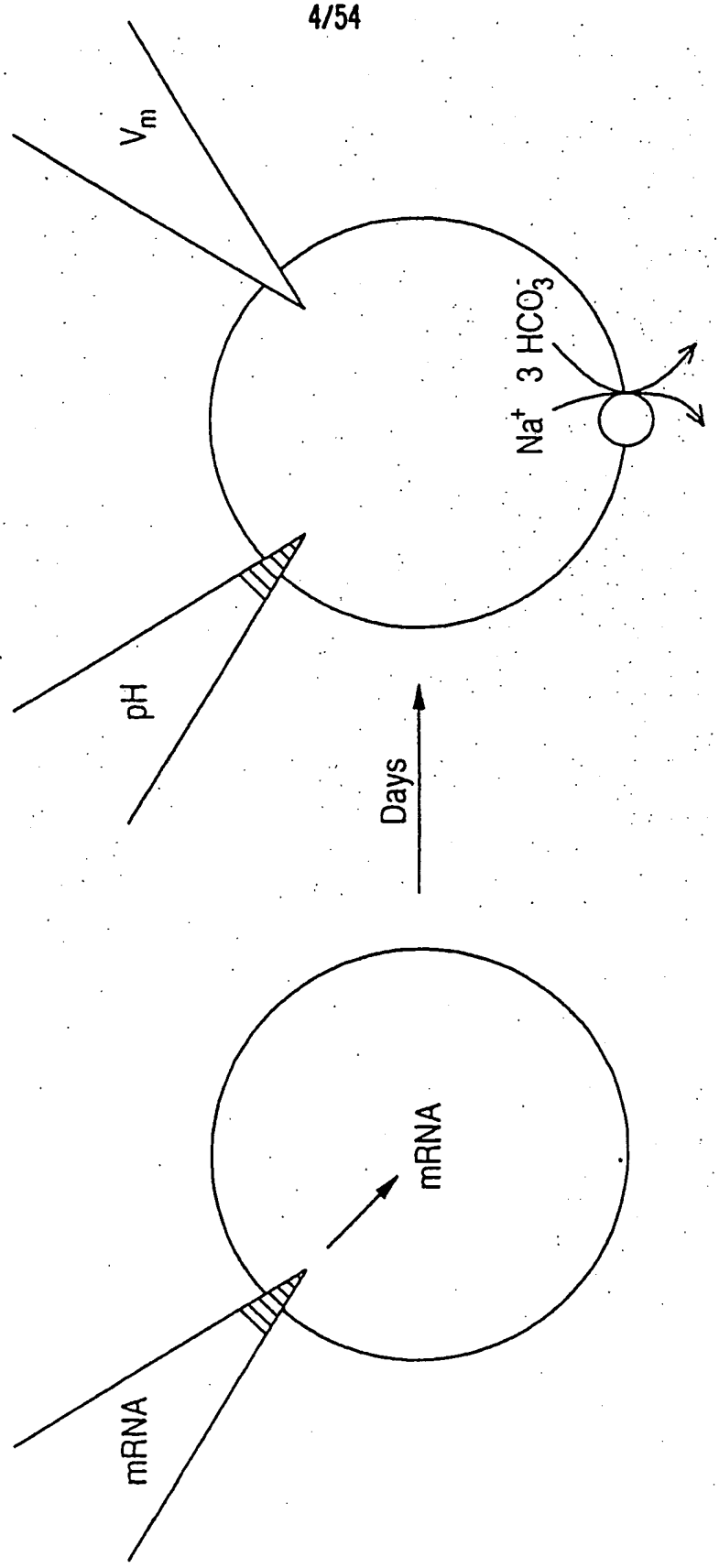
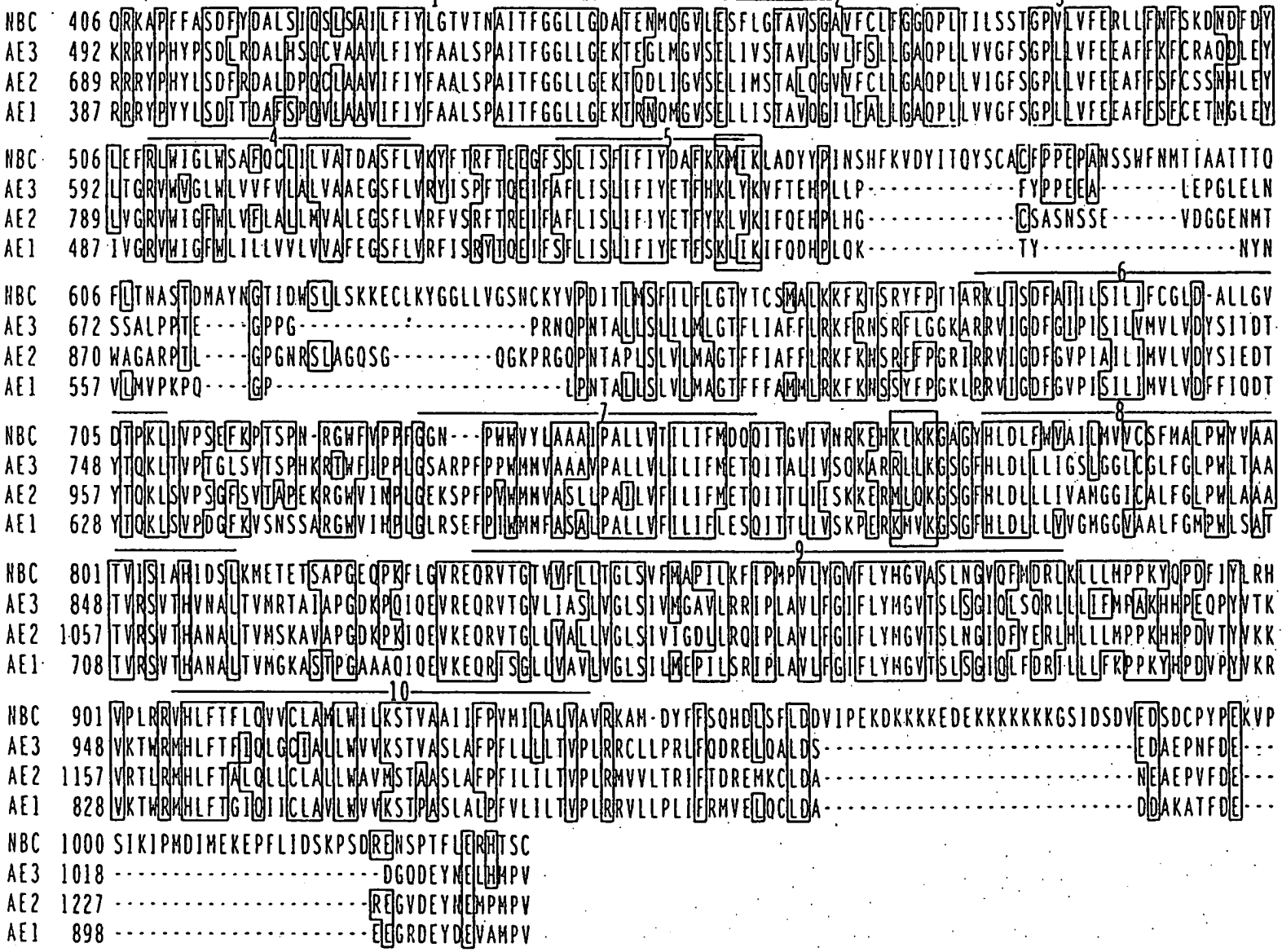



FIG. 4

FIG. 5A1

## FIG. 5A2


1
2
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10
NBC 406 QRKAPFFASDFYDALSIQSLSAILFIYLGTVTNAITFGGLLGDATENMQGVLESFLGTAVSGAVFCLFGGQPLTILSSTGPVLVFERLLFNFSKDNDFDY
AE3 492 KRRYPHYPSDLRDALHSQCVAAAVLFIYFAALSPAITFGGLLGEKTEGLMGVSELIVSTAVLGVLFSLLGAQPLLVVGFSGPLLVFEEAFFKFCRAQDLEY
AE2 689 RRRYPHYLSDFRDALDPQCLAAAVIFIYFAALSPAITFGGLLGEKTQDLIGVSELIMSTALQGVVFCLLGAQPLLVIGFSGPLLVFEEAFFSFCSSNHLEY
AE1 387 RRRYPYYLSDITDAFSPQVLAAAVIFIYFAALSPAITFGGLLGEKTRNQMGVSELLISTAVQGILFALLGAQPLLVVGFSGPLLVFEEAFFSFCETNGLEY
NBC 506 LEFRLWIGLWSAFQCLILVATDASFLVKYFTRFTEEGFSSLISFIFIYDAFKKKMIKLADYYPINSHFKVDYITQYSCACFPPEPANSSWFNMTTAATTTQ
AE3 592 LTGRVWVGLWLVVFVLALVAAEGSFLVRYISPFTQEIFAFLISLIFIYETFHKLYKVFTEHPLLP----------FYPPEEA-------LEPGLELN
AE2 789 LVGRVWIGFWLVFLALLMVALEGSFLVRFVSRFTREIFAFLISLIFIYETFYKLVKIFQEHPLHG----------CSASNSSE------VDGGENMT
AE1 487 IVGRVWIGFWLILLVVLVVAFEGSFLVRFISRYTQEIFSFLISLIFIYETFSKLIKIFQDHPLQK----------TY----------NYN
NBC 606 FLTNASTDMAYNGTIDWSLLSKKECLKYGGLLVGSNCKYVPDITLMSFILFLGTYTCSMALKKFKTSRYFPTTARKLISDFAIILSILIFCGLD-ALLGV
AE3 672 SSALPPTE----GPPG---------PRNQPNTALLSLILMLGTFLIAFFLRKFRNSRFLGGKARRVIGDFGIPISILVMVLVDYSITDT
AE2 870 WAGARPTL----GPGNRSLAGQSG---------QGKPRGQPNTAPLSLVLMAGTFFIAFFLRKFKHSRFFPGRIRRVIGDFGVPIAILIMVLVDYSIEDT
AE1 557 VLMVPKPQ----GP----------LPNTALLSLVLMAGTFFFAMMLRKFKNSSYFPGKLRRVIGDFGVPISILIMVLVDFFIQDT
NBC 705 DTPKLIVPSEFKPTSPN-RGWFVPPFGGN---PWWVYLAAAIPALLVTILIFMDQQITGVIVNRKEHKLKKGAGYHLDLFWVAILMVVCSFMALPHYVAA
AE3 748 YTQKLTVPTGLSVTSPHKRTWFIPPLGSARPFPPWMNVAAAMPALLVLILIFMETQITALIVSQKARRLLKGSGFHLDLLLIGSLGGLCGLFGLPWLTAA
AE2 957 YTQKLSVPSGFSVTAPEKRGWVINPLGEKSPFPVWMNVASLLPAILVFILIFMETQITTLIISKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAA
AE1 628 YTQKLSVPDGFKVSNSSARGWVIHPLGLRSEFPIWMMFASALPALLVFILIFLESQITTLIVSKPERKMVKGSGFHLDLLLVVGMGGVAALFGMPWLSAT
NBC 801 TVISIAHIDSLKMETETSAPGEQPKFLGVREQRVTGTVVFLLTGLSVFMAPILKFIPMPVLYGVFLYHGVASLNGVQFMDRLKLLLMPPKYQPDFIYLRH
AE3 848 TVRSVTHVNALTVMRTAIAPGDKPQIQEVREQRVTGVLIASLVGLSIVMGAVLRRIPLAVLFGIFLYMGVTSLSGIQLSQRLLLIFMPAKHHPEQPYVTK
AE2 1057 TVRSVTHANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVAALLVGLSIVIGDLLRQIPLAVLFGIFLYMGVTSLNGIQFYERLHLLLMPPKHHPDVTYVKK
AE1 708 TVRSVTHANALTVMGKASTPGAAAQIQEVKEQRISGLLVAVLVGLSILMEPILSRIPLAVLFGIFLYMGVTSLSGIQLFDRILLLFKPPKYHPDVPYVKR
NBC 901 VPLRRVHLFTFLQVVCLAMLWILKSTVAAIIFPVMILALVAVRKAM-DYFFSQHDLSFLDDVIPEKDKKKKEDEKKKKKKKGSIDSDVEDSDCPYPEKVP
AE3 948 VKTWRMHLFTFIQLGCIALLWVVKSTVASLAFPFLLLLTVPLRRCLLPRLFQDRELQALDS----------EDAEPNFDE---
AE2 1157 VRTLRMHLFTALQLLCLALLWAVMSTAASLAFPFILILTVPLRMVVLTRIFTDREMKCLDA----------NEAEPVFDE---
AE1 828 VKTWRMHLFTGIQIICLAVLWVVKSTPASLALPFVLILTVPLRRVLLPLIFRMVELQCLDA----------DDAKATFDE---
NBC 1000 SIKIPMDIMEKEPFLIDSKPSDRENSPTFLERHTSC
AE3 1018 ----------DGQDEYMELHMPV
AE2 1227 ----------REGVDEYMEMPMPV
AE1 898 ----------EEGRDEYDEVAMPV

FIG. 5B

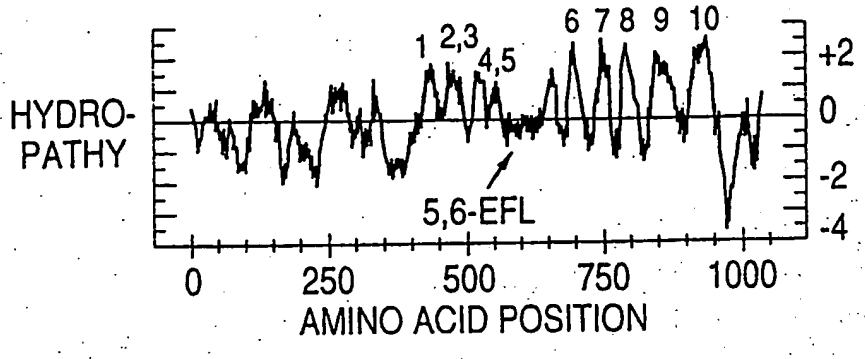



FIG. 5C

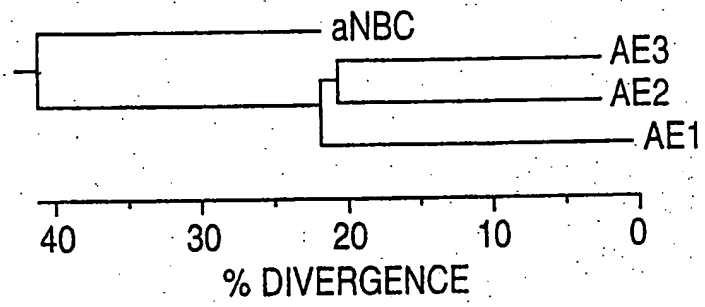



FIG. 5D

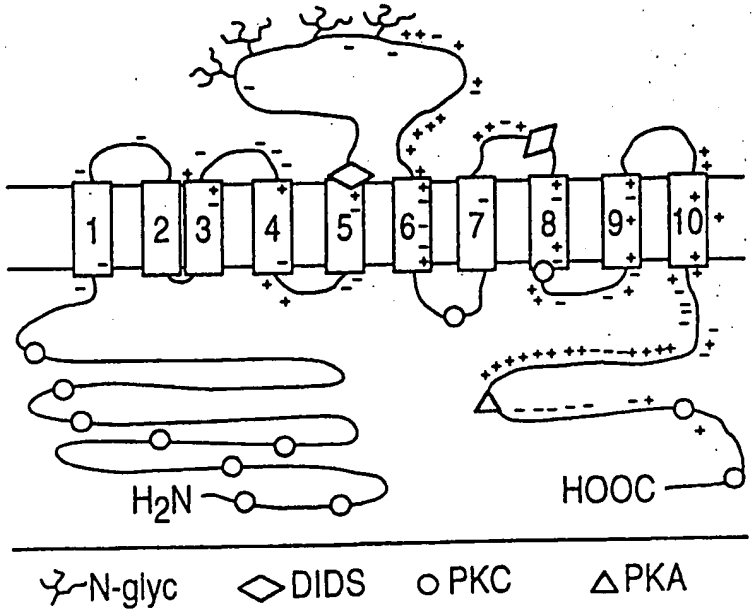


FIG. 6

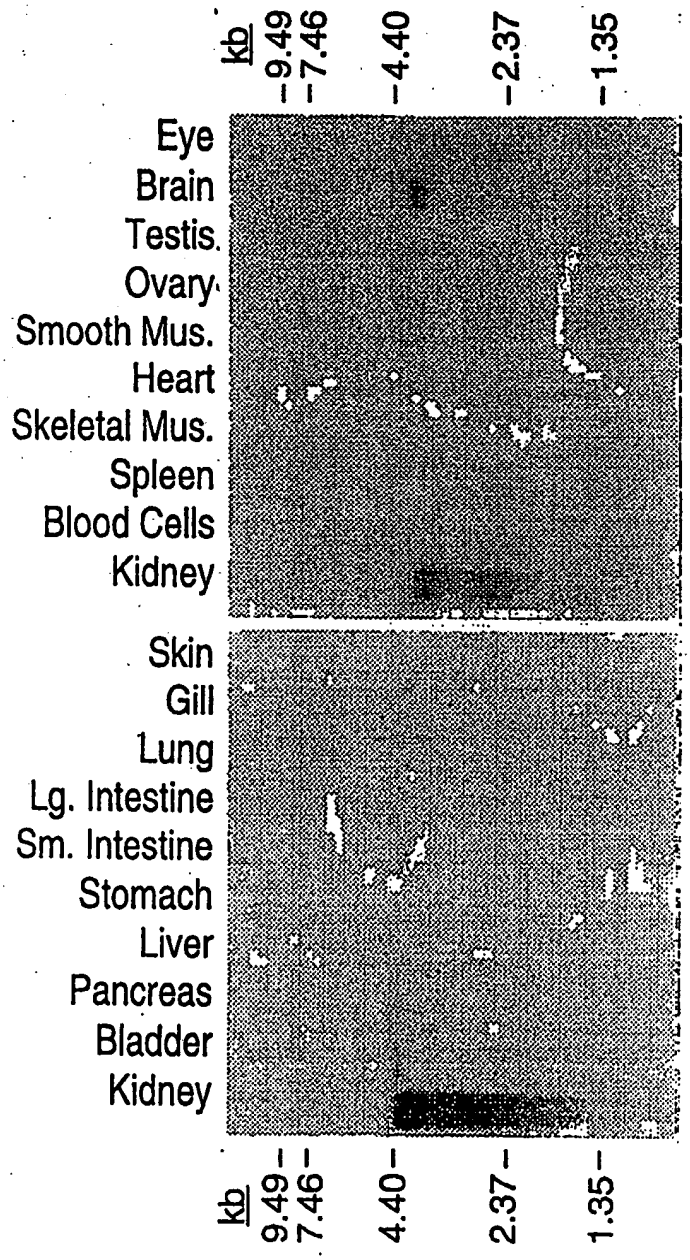

kb
9.49
7.46
4.40
2.37
1.35
Eye
Brain
Testis.
Ovary
Smooth Mus.
Heart
Skeletal Mus.
Spleen
Blood Cells
Kidney
Skin
Gill
Lung
Lg. Intestine
Sm. Intestine
Stomach
Liver
Pancreas
Bladder
Kidney
kb
9.49
7.46
4.40
2.37
1.35

FIG. 7A

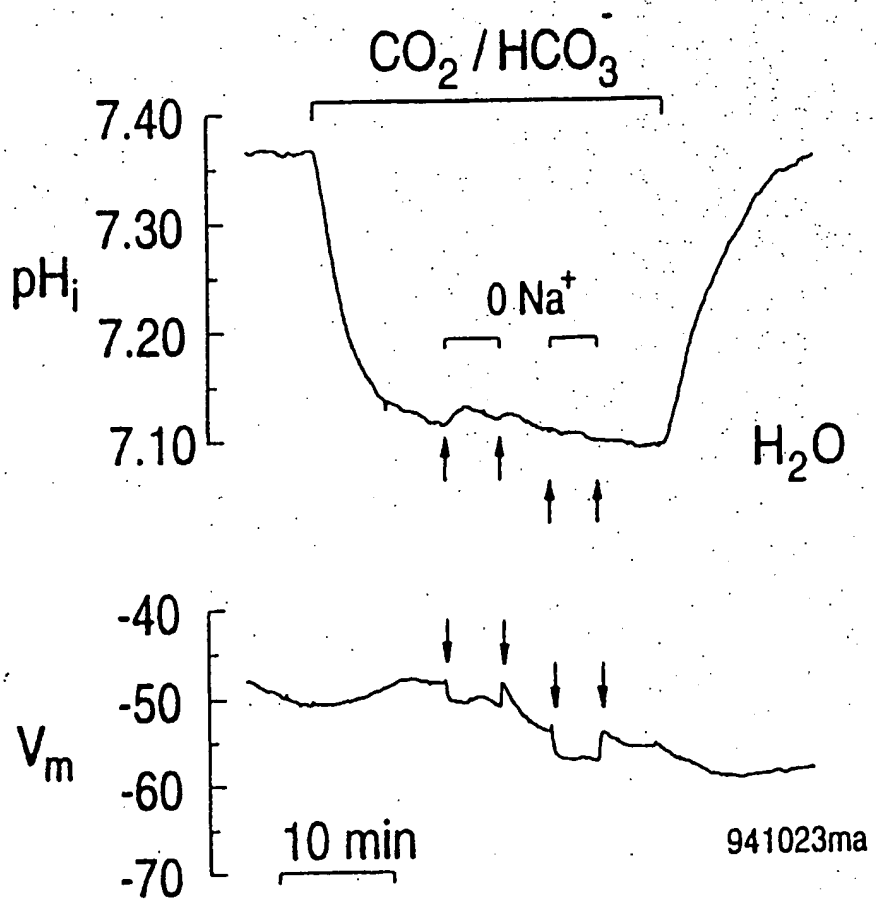

$CO_2 / HCO_3^-$
7.40
7.30
7.20
7.10
$pH_i$
0 $Na^+$
$H_2O$
-40
-50
-60
-70
$V_m$
10 min
941023ma

FIG. 7B

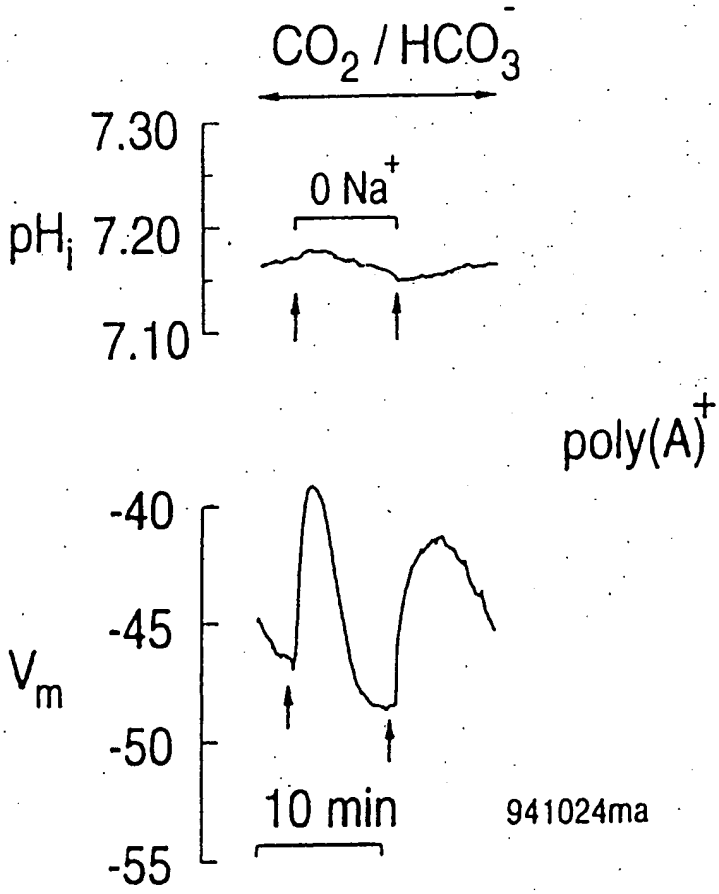

$CO_2$ / $HCO_3^-$
7.30
$pH_i$ 7.20
7.10
0 $Na^+$
poly(A)$^+$
-40
-45
$V_m$
-50
-55
10 min
941024ma

FIG. 7C

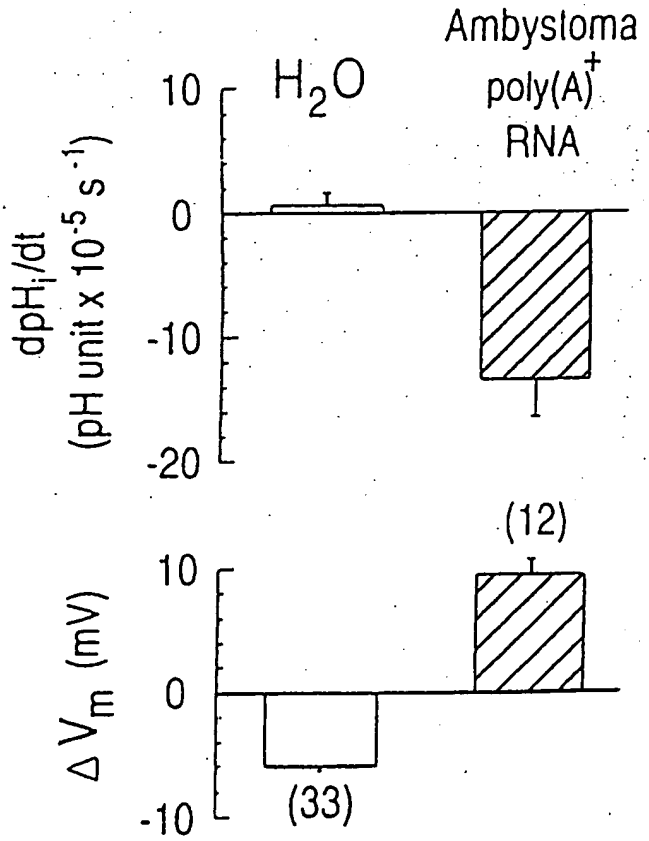

Ambystoma
poly(A)+
RNA
H2O
10
0
-10
-20
dpHi/dt
(pH unit x 10-5 s-1)
(12)
10
0
-10
ΔVm (mV)
(33)

FIG. 8A

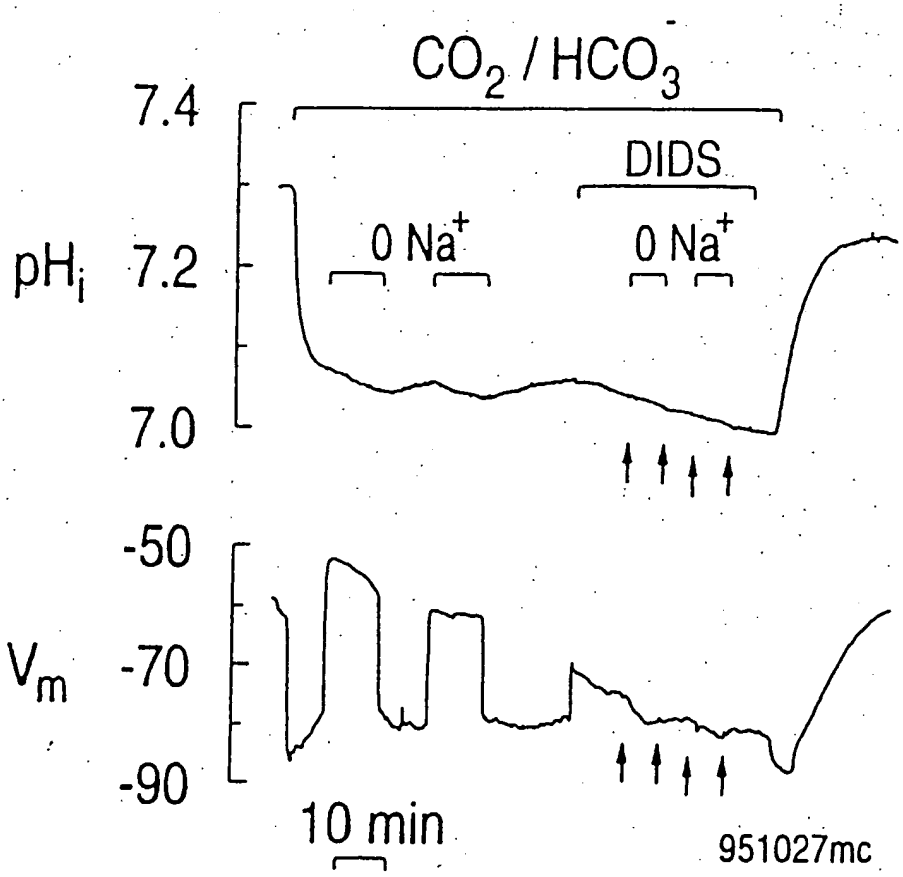
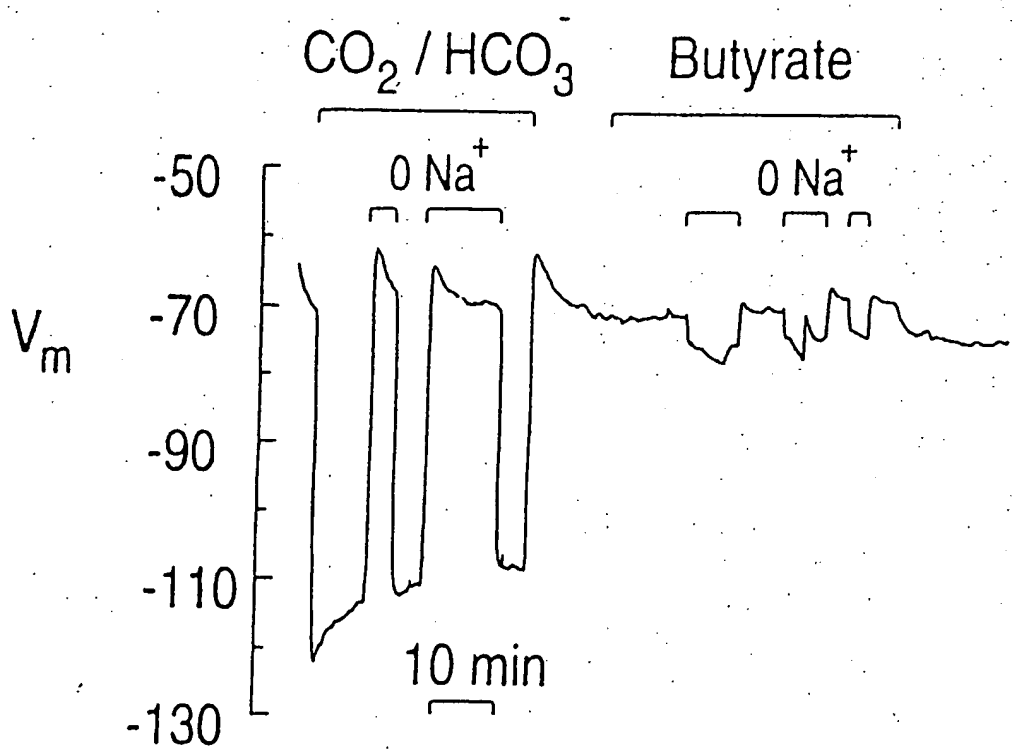


FIG. 8B




960807mb

FIG. 8C

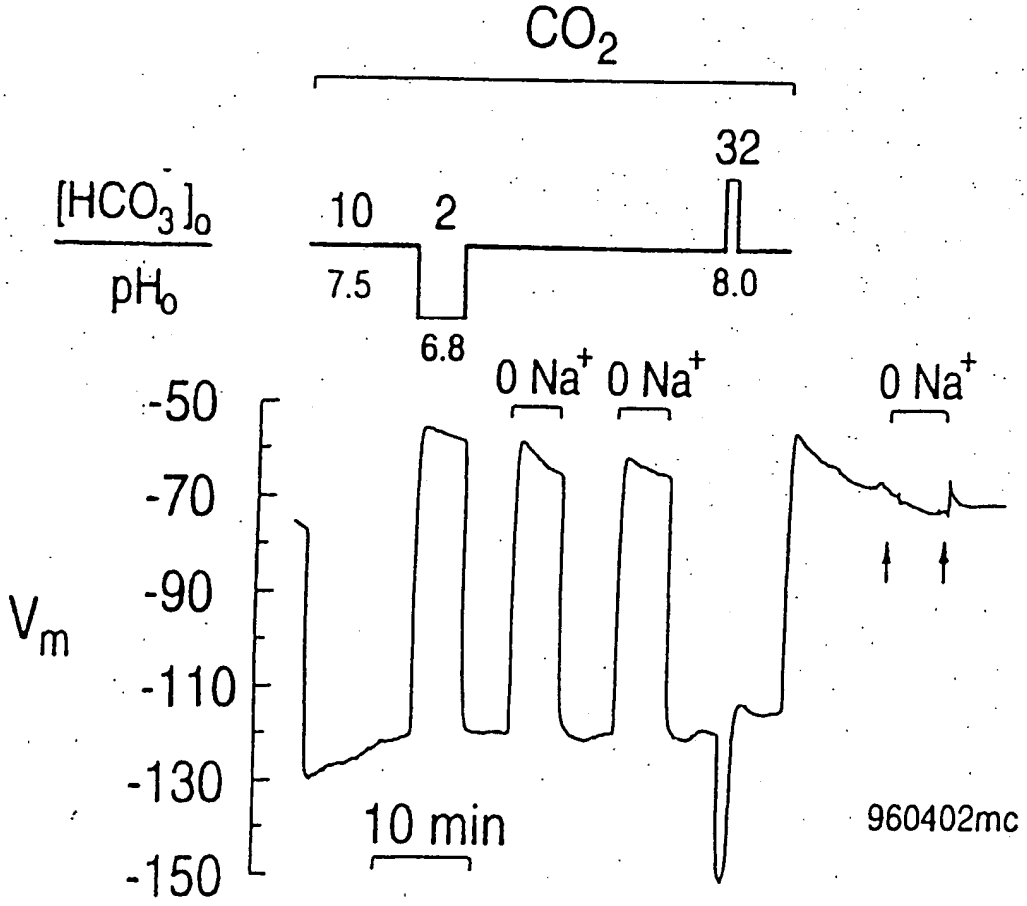

$CO_2$
32
$[HCO_3^-]_o$
10
2
$pH_o$
7.5
6.8
8.0
0 $Na^+$
0 $Na^+$
0 $Na^+$
-50
-70
-90
-110
-130
-150
$V_m$
10 min
960402mc

FIG. 8D

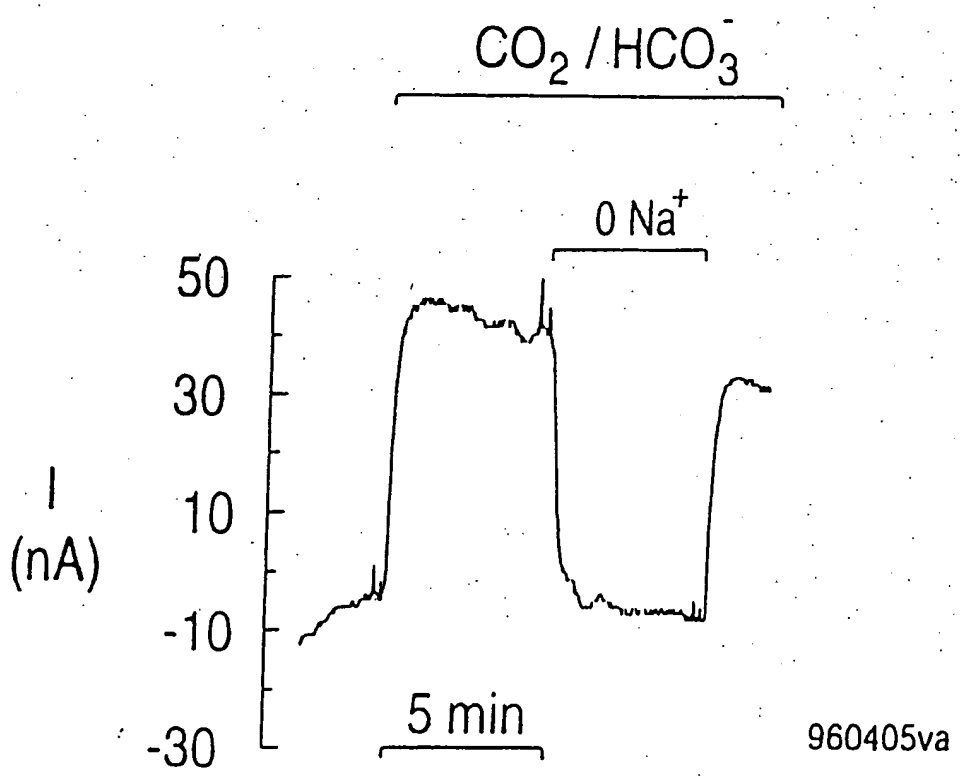


FIG. 9(1)

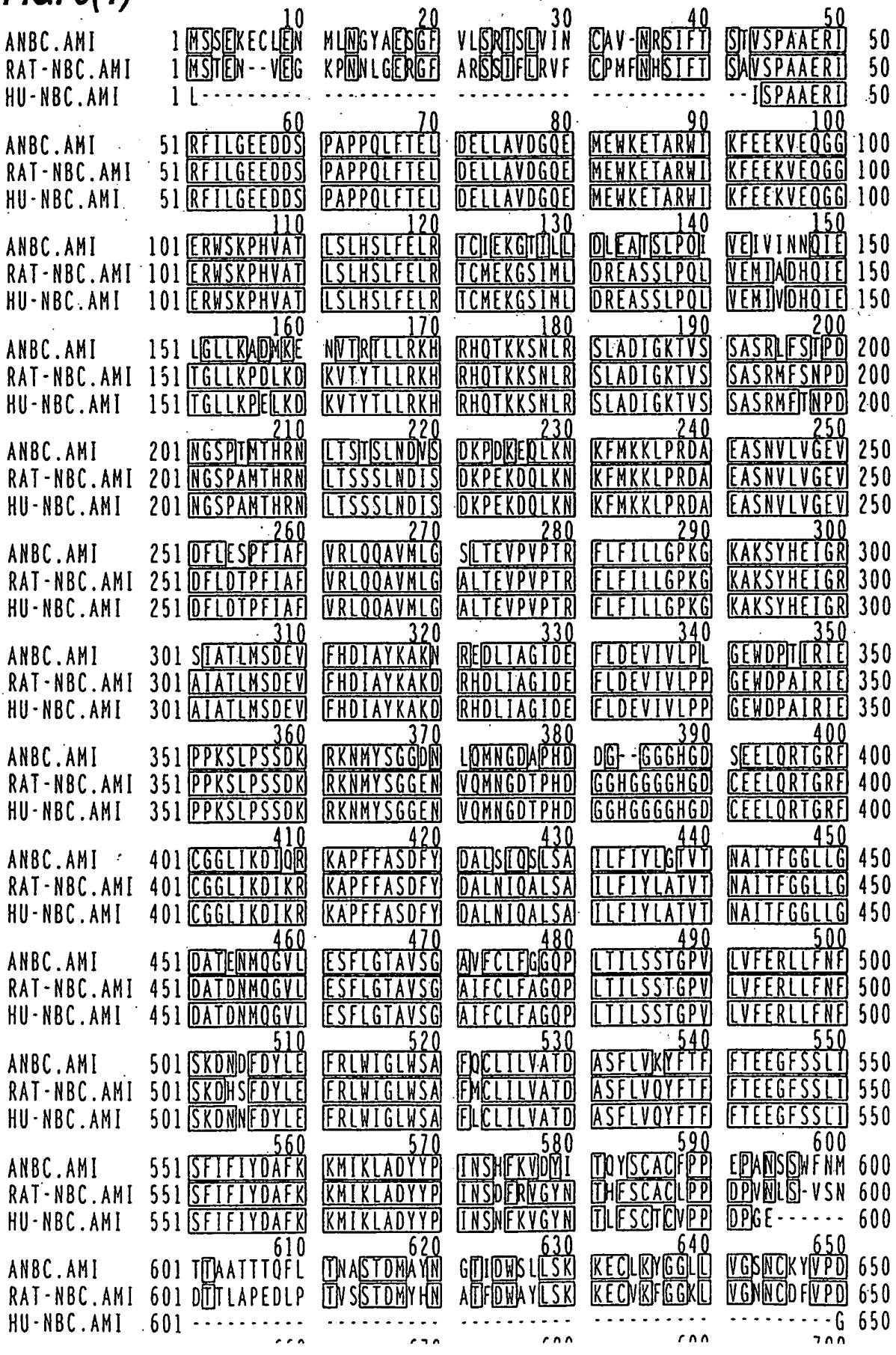
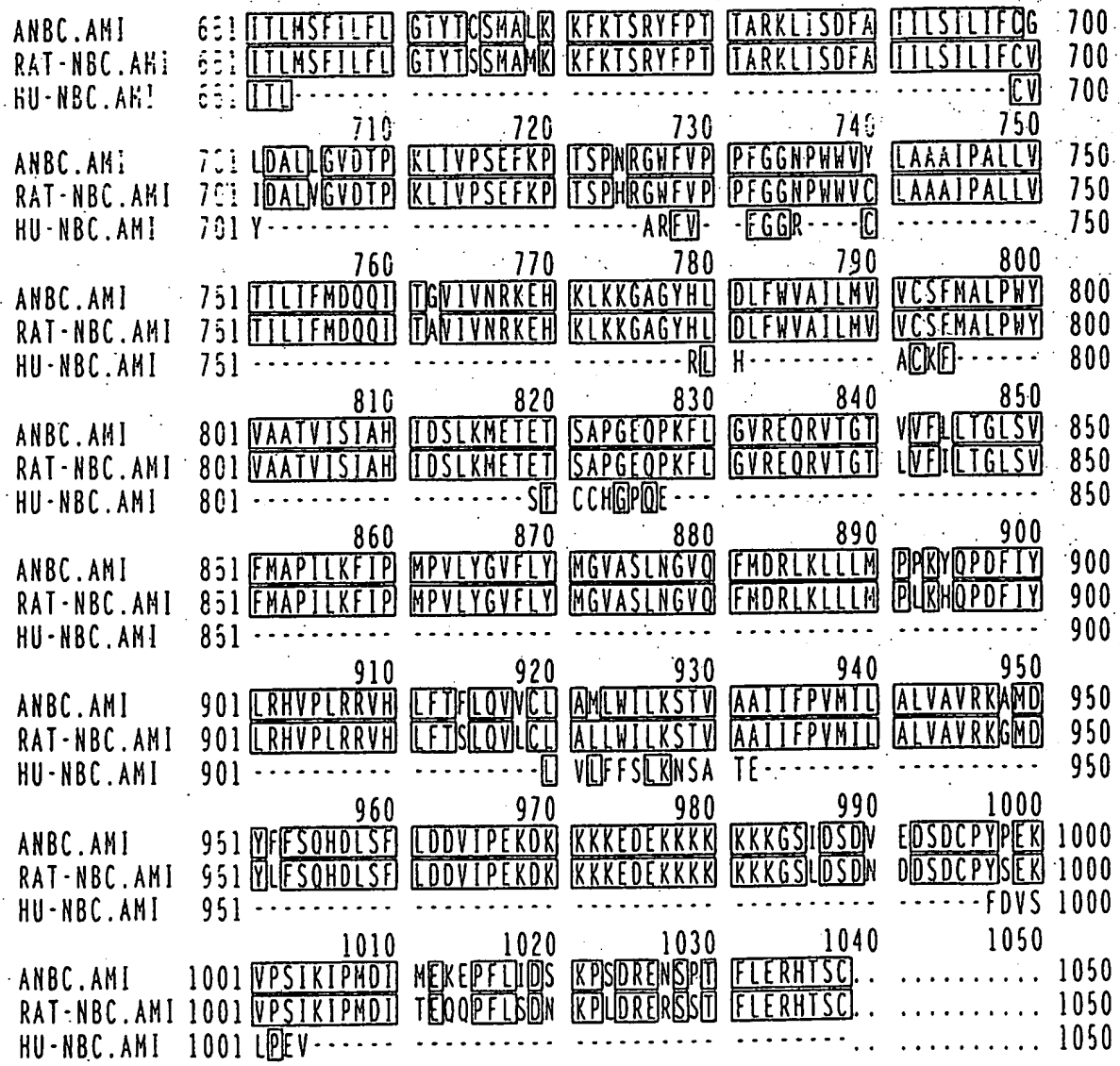


*FIG. 9(2)*


ANBC.AMI 681 ITLMSFILFL GTYTCSMALK KFKTSRYFPT TARKLISDFA IILSILIFCG 700
RAT-NBC.AMI 681 ITLMSFILFL GTYTSSMAMK KFKTSRYFPT TARKLISDFA IILSILIFCV 700
HU-NBC.AMI 681 ITL------- ---------- ---------- ---------- --------CV 700
710 720 730 740 750
ANBC.AMI 701 LDALLGVDTP KLIVPSEFKP TSPNRGWFVP PFGGNPWWVY LAAAIPALLV 750
RAT-NBC.AMI 701 IDALVGVDTP KLIVPSEFKP TSPHRGWFVP PFGGNPWWVC LAAAIPALLV 750
HU-NBC.AMI 701 Y--------- ---------- -----ARFV- -FGGR----C ---------- 750
760 770 780 790 800
ANBC.AMI 751 TILIFMDQQI TGVIVNRKEH KLKKGAGYHL DLFWVAILMV VCSFMALPWY 800
RAT-NBC.AMI 751 TILIFMDQQI TAVIVNRKEH KLKKGAGYHL DLFWVAILMV VCSFMALPWY 800
HU-NBC.AMI 751 ---------- ---------- --------RL H--------- ACKF------ 800
810 820 830 840 850
ANBC.AMI 801 VAATVISIAH IDSLKMETET SAPGEQPKFL GVREQRVTGT VVFLLTGLSV 850
RAT-NBC.AMI 801 VAATVISIAH IDSLKMETET SAPGEQPKFL GVREQRVTGT LVFILTGLSV 850
HU-NBC.AMI 801 ---------- --------ST CCHGPQE--- ---------- ---------- 850
860 870 880 890 900
ANBC.AMI 851 FMAPILKFIP MPVLYGVFLY MGVASLNGVQ FMDRLKLLLM PPKYQPDFIY 900
RAT-NBC.AMI 851 FMAPILKFIP MPVLYGVFLY MGVASLNGVQ FMDRLKLLLM PLKHQPDFIY 900
HU-NBC.AMI 851 ---------- ---------- ---------- ---------- ---------- 900
910 920 930 940 950
ANBC.AMI 901 LRHVPLRRVH LFTFLQVVCL AMLWILKSTV AAIIFPVMIL ALVAVRKAMD 950
RAT-NBC.AMI 901 LRHVPLRRVH LFTSLQVLCL ALLWILKSTV AAIIFPVMIL ALVAVRKGMD 950
HU-NBC.AMI 901 ---------- ---------L VLFFSLKNSA TE-------- ---------- 950
960 970 980 990 1000
ANBC.AMI 951 YFFSQHDLSF LDDVIPEKDK KKKEDEKKKK KKKGSIDSDV EDSDCPYPEK 1000
RAT-NBC.AMI 951 YLFSQHDLSF LDDVIPEKDK KKKEDEKKKK KKKGSLDSDN DDSDCPYSEK 1000
HU-NBC.AMI 951 ---------- ---------- ---------- ---------- ------FDVS 1000
1010 1020 1030 1040 1050
ANBC.AMI 1001 VPSIKIPMDI MEKEPFLIDS KPSDRENSPT FLERHTSC.. .......... 1050
RAT-NBC.AMI 1001 VPSIKIPMDI TEQQPFLSDN KPLDRERSST FLERHTSC.. .......... 1050
HU-NBC.AMI 1001 LPEV------ ---------- ---------- --------.. .......... 1050

# FIG. 10(1)

TO FIG. 10(2)

```
Rat-kidney NBC: ...AAAGGAAGTCTGGATAGCGACAACGACGATTCTGACT
rbNBC:          ...AAAGGAAGTCTGGATAGCGACAACGACGAT-------

Rat-kidney NBC:  CAGCAACCTTTCCTAAGTGATAACAAACCCTTGGACA
rbNBC:           ------------------------------------

Rat-kidney NBC:  CTTTCCTTGAGTCACTGGGTTTACCCAGTCCTCCAAG
rbNBC:           CTTTCCTTGAGTCACTGGGTTTACCCAGTCCTCCAAG

Rat-kidney NBC:  AACGATTATCTTTGGAGGAACAAGGGAACAGAAACCA
rbNBC:           AACGATTATCTTTGGAGGAACAAGGGAACAGAAACCA
```

Rat-kidney NBC: ...KGSLDSDNDDSDCPYSEKVPSIKIPMDITEQ

Novel isoform: ...KGSLDSDNDD**EKDPQHSLNATHHADKIPFLE**

TO FIG. 10(2)

# FIG. 10(2)

← TO FIG. 10(1)

GCCCATACTCAGAAAAGGTCCCCAGTATTAAAATTCCAATGGCATCACGGAA

GAGAAAGATCCTCAACATTCCTTGAACGCCACACATCATGCTGATAAAATTC
GAGAAAGATCCTCAACATTCCTTGAACGCCACACATCATGCTGATAAAATTC

ATCTCCAGTGAAAGTCGTGCCTCAAATTAGAATAGAACTTGAGTCTGAAGAC
ATCTCCAGTGAAAGTCGTGCCTCAAATTAGAATAGAACTTGAGTCTGAAGAC

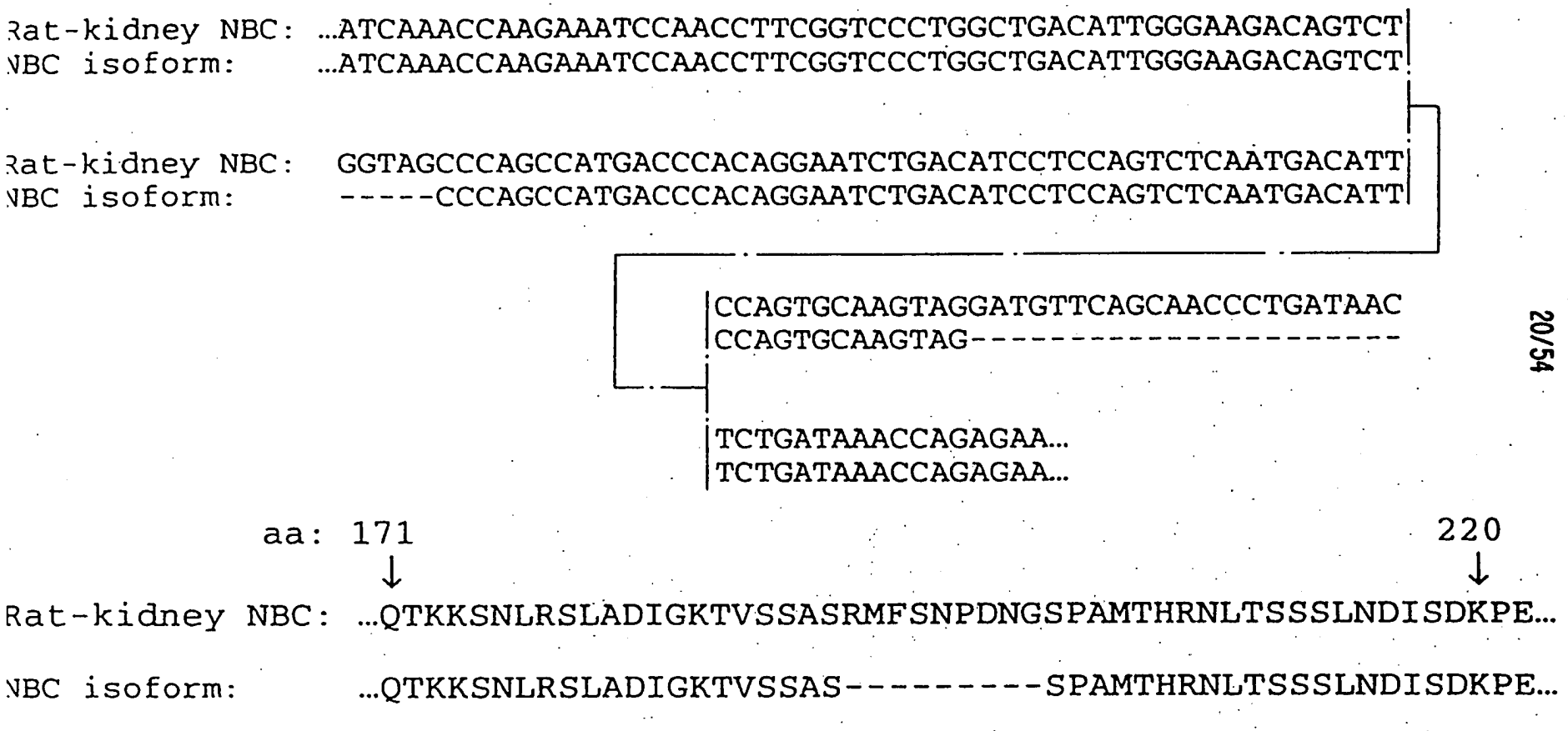
CGTTGTAA
CGTTGTAA

QPFLSDNKPLDRERSSTFLERHTSC

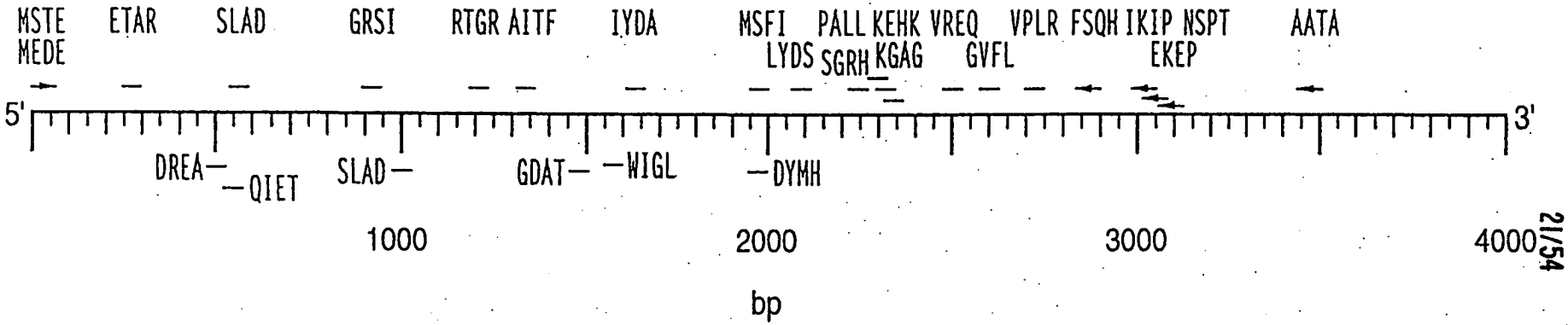
**SLGLPSPPRSPVKVVPQIRIELESEDNDYLWRNKGTETTL**

← TO FIG. 10(1)

FIG. 11


Rat-kidney NBC: ...ATCAAACCAAGAAATCCAACCTTCGGTCCCTGGCTGACATTGGGAAGACAGTCT
NBC isoform: ...ATCAAACCAAGAAATCCAACCTTCGGTCCCTGGCTGACATTGGGAAGACAGTCT
Rat-kidney NBC: GGTAGCCCAGCCATGACCCACAGGAATCTGACATCCTCCAGTCTCAATGACATT
NBC isoform: -----CCCAGCCATGACCCACAGGAATCTGACATCCTCCAGTCTCAATGACATT
CCAGTGCAAGTAGGATGTTCAGCAACCCTGATAAC
CCAGTGCAAGTAG----------
TCTGATAAACCAGAGAA...
TCTGATAAACCAGAGAA...
aa: 171
220
Rat-kidney NBC: ...QTKKSNLRSLADIGKTVSSASRMFSNPDNGSPAMTHRNLTSSSLNDISDKPE...
NBC isoform: ...QTKKSNLRSLADIGKTVSSAS---------SPAMTHRNLTSSSLNDISDKPE...

# FIG. 12




NBC PCR Primers

Except where noted by single arrow primers are sense and antisense

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FIG. 13(1)

TO FIG. 13(2)

| | |
|---|---|
| ATGGAGGATGAAGCTGTCCTGGACAGAGGG | hMEDE_FWD |
| ATGTCCACTGAAAATGTGGAAGGGAAGCCC | rMSTE_FWD |
| CCCGGACCGGCGGCCGCATGTCCACTGAAAATGTGGAAGGGAAGCCC | rMSTE_RN_FWD |
| GAGACAGCCAGGTGGATTAAGTTTGAAGAG | rETAR_FWD |
| AGATCTCGAGCGGCCGCCCGAGACAGCCAGGTGGATTAAG | RETARNOT_FWD |
| CTCTTCAAACTTAATCCACCTGGCTGTCTC | rETAR_REV |
| AGGTTTAGGTTTTAGGATGCTGCTGCTGGA | hSSSS_REV |
| TCCAGCAGCAGCATCCTAAAACCTAAACCT | hSSSS_FWD |
| GAAACAGCCAGGTGGATCAAGTTTGAAGAA | hETAR_FWD |
| GTGCCCACAAGGTTCTTGTTCATTCTCTTA | hVPTR_FWD |
| TAAGAGAATGAACAAGAACCTTGTGGGCAC | hVPTR_REV |
| TTCTTCAAACTTGATCCACCTGGCTGTTTC | hETAR_REV |
| CGAACTGGACGGTTCTGTGGTGGACTAATT | hRTGR_FWD |
| AATTAGTCCACCACAGAACCGTCCAGTTCG | hRTGR_REV |
| GCTATCACTTTTGGAGGACTGCTTGGGGAT | hAITF_FWD |
| ATCCCCAAGCAGTCCTCCAAAAGTGAT | hAITF_REV |
| AGAATCTCGAGCGGCCGCCGAAACAGCCAGGTGGATCAAGTTTGAAGAA | hETAR_NT_FWD |
| TCCCTGGCTGACATTGGGAAGACA | hSLAD_FWD |
| TGTCTTCCCAATGTCAGCCAGGGA | hSLAD_REV |
| GGCAGAGCCATTGCCACCCTGATGTCTGAT | hGRAI_FWD |
| ATCAGACATCAGGGTGGCAATGGCTCTGCC | hGRAI_REV |
| TAGATCCGCGAGCGGCCGCCCCATCAGACATCAGGGTGGCAATGGCTCT | hGRAI_NT_REV |
| ATCTATGATGCTTTCAAGAAGATGATCAAG | hIYDA_FWD |
| CTTGATCATCTTCTTGAAAGCATCATA | hIYDA_REV |
| CACAAAGCGTAATGCCCTCACCT | hDPEG_REV |
| CCAAATACAAAGCGAGCATAAACA | hVYAR_REV |
| AGCAGCATCCTAAAACCTCTCATCTCTCCT | hSSIL_FWD |
| ATGTCTTTTATCCTCTTCTTGGGAACCATG | hMSFI_FWD |
| CATGGTTCCCAAGAAGAGGATAAAAGACAG | hMSFI_REV |
| CCGGCTTTGTTGGCTACTATACTGATTTTC | hPALL_FWD |
| GAAAATCAGTATAGTGACCAACAAAGCCCG | hPALL_REV |
| AAAGAACATAAACTCAAGAAAGGAGCAGGG | hKEHK_FWD |
| AAAGAGATCCAAGTGATACCCTGCTCCTTT | hKGAG_REV |
| GGTGTGTTCCTGTATATGGGAGTAGCATCC | hGVFL_FWD |
| GGATGCTACTCCCATATACAGGAACACACC | hGVFL_REV |
| GTTCCTCTGGCGAGAGTCCACCTGTTCACT | hVPLR_FWD |
| AGTGAACAGGTGGACTCTGCGCAGAGGAAC | hVPLR_REV |
| TCAGCATGATGTGTGGCGTTCAAGGAATGT | r/hTFLE_REV |
| TCCCTGGCTGACATTGGGAAGACAGTCTCC | rSLAD_FWD |
| GGAGACTGTCTTCCCAATGTCAGCCAGGGA | rSLAD_REV |
| GTGCCCACAAGGTTCTTGTTCATTCTCTTA | rVPTR_FWD |
| TAAGAGAATGAACAAGAACCTTGTGGGCAG | rVPTR_REV |
| GGAAGAGCCATCGCCACCTTGATGTCTGAC | rGRSI_FWD |
| GTCAGACATCAAGGTGGCGATGGCTCTTCC | rGRSI_REV |
| TAGATCGCGAGCGGCCGCCCAGACATCAAGGAGGCGATGGCTCTTCC | rGRSI_NT_REV |
| AGAACTGGCCGGTTCTGTGGTGGATTAATT | rRTGR_FWD |
| AATTAATCCACCACAGAACCGGCCAGTTCT | rRTGR_REV |
| GCCATCACTTTCGGAGGCCTGCTCGGGGAT | rAITF_FWD |
| GCCATCACTTTCGGAGGCCTGCTCGGGGAT | rAITF_REV |

TO FIG. 13(2)

# FIG. 13(2)

← TO FIG. 13(1)

| | |
|---|---|
| 1 - 30 bp | |
| 1-30 BP | |
| rMSTE_FWD WITH RsrII/NotI RE | |
| 247-276 | |
| ETAR_FWD WITH A NOTI SITE BUILT IN | |
| 247-276 | |
| 293-270 | |
| 270-293 | |
| 423-452 | |
| 999-1028 | |
| 1028-999; REV COMPLEMENT TO FWD | |
| REVERSE COMPLEMENT TO hETAR_FWD | |
| 1310-1339 | |
| REC COMP TO RTGR_FWD | |
| 1448-1477 | |
| REV COMP TO FWD | |
| 423-452 PLUS A NOTI RE SITE | |
| 665-694 | |
| REV COMP TO hSLAD_FWD | |
| 1019-1048; NOTE THIS WAS ORIGINALLY 'GRSI' AS THE FIRST SEQ WERE GIVEN aNBC | |
| REV COMP TO GRAI_FWD | |
| REV COMP TO GRAI_FWD WITH A NOTI SITE ADDED | |
| 1787-1816; THIS SEQUENCE IS THE SAME IN RAT WILL BE REFERD TO AS r/hIYDA | |
| REV COMP TO hIYDA_FWD | |
| 1899-1923 HUMAN NBC (MFR DESIGN) | |
| 1922-1944 3'END OF hNBC (MFR DESIGN) | |
| 232-261 | |
| 2085-2112; SYN 8/13/97 | |
| 2085-2112; SYN 8/13 | |
| 2359-2388; SYN 8/13 | |
| 2359-2388; RESYN 8/18/97 | |
| 2425-2454; 8/14/97 | |
| 2443-2472 | |
| 2719-2748; SYN 8/14/97 | |
| 2719-2748 | |
| 2833-2862; 8/14/97 | |
| 2833-2862; 8/14/97 | |
| 3211-3240; 3'END w/STOP CODON; SYN 8/14/97 | |
| 535-564 | |
| 535-564 | |
| 823-852 | |
| 823-852 | |
| 899-918 | |
| 899-918 | |
| rGRSI_REV WITH A NOTI RE SITE ON 5'END | |
| 1180-1209 | |
| | |
| 1318-1347 | |
| | |

← TO FIG. 13(1)

FIG. 14

PAC DNA PCR PRODUCTS USING HUMAN NBC PRIMERS TO PREDICT REGIONS FOR INTRONS

| CLONE I.D. | Forward Primer | Reverse Primer | Size of Intron(s) (1) (base pair) |
|---|---|---|---|
| 839 | SSSS | DPGE | 3000 |
| | RTGR | VPLR | 1000 |
| | GVFL | TFLE | 9000 |
| | PALL | KGAG | 6000 |
| 864 | GVFL | TFLE | 9000 |
| | PALL | KGAG | 1000 |
| 929 | SSSS | DPGE | 4500 |
| | RTGR | VPLR | 1000 |
| | GVFL | TFLE | 6000 |
| | PALL | KGAG | 6500 |
| 947 | SSSS | DPGE | 5000 |
| | RTGR | VPLR | 1000 |
| | ETAR | QIET | 6000 |
| | GVGL | TFLE | 9000 |
| | PALL | KGAG | 6000 |
| 15256 | GRAI | AITF | 1000 |
| | AITF | MSFI | 800 |
| | RTGR | VPLR | 1000 |
| 15257 | ETAR | GRSI | 300 |

See Table I for sequence of these primers.

Note 1. This value is the approximate # of base pairs above the size of the PCR product predicted for NBC cDNA

FIG. 15A1

AGGAGAAGTCTGAGCATTCTTTTTTTCTTTTTTATTACTATAGGATGGAGGATGAAGCTGTCCTGGACAGAG
GGGCTTCCTTCCTCAAGCATGTGTGTGATGAAGAAGAAGTAGAAGGCCACCATACCATTTACATCGGAGTCC
ATGTGCCGAAGAGTTACAGGAGAAGGAGACGTCACAAGAGAAAGACAGGGCACAAAGAAAAGAAGGAAAAGG
AGAGAATCTCTGAGAACTACTCTGACAAATCAGATATTGAAAATGCTGATGAATCCAGCAGCAGCATCCTAA
AACCTCTCATCTCTCCTGCTGCAGAACGCATCCGATTCATCTTGGGAGAGGAGGATGACAGCCCAGCTCCCC
CTCAGCTCTTCACGGAACTGGATGAGCTGCTGGCCGTGGATGGGCAGGAGATGGAGTGGAAGGAAACAGCCA
GGTGGATCAAGTTTGAAGAAAAAGTGGAACAGGGTGGGGAAAGATGGAGCAAGCCCCATGTGGCCACATTGT
CCCTTCATAGTTTATTTGAGCTGAGGACATGTATGGAGAAAGGATCCATCATGCTTGATCGGGAGGCTTCTT
CTCTCCCACAGTTGGTGGAGATGATTGTTGACCATCAGATTGAGACAGGCCTATTGAAACCTGAACTTAAGG
ATAAGGTGACCTATACTTTGCTCCGGAAGCACCGGCATCAAACCAAGAAATCCAACCTTCGGTCCCTGGCTG
ACATTGGGAAGACAGTCTCCAGTGCAAGTAGGATGTTTACCAACCCTGATAATGGTAGCCCAGCCATGACCC
ATAGGAATCTGACTTCCTTCAGTCTGAATGACATTTCTGATAAACCGGAGAAGGACCAGCTGAAGAATAAGT
TCATGAAAAAATTGCCACGTGATGCAGAAGCTTCCAACGTGCTTGTTGGGGAGGTTGACTTTTTGGATACTC
CTTTCATTGCCTTTGTTAGGCTACAGCAGGCTGTCATGCTGGGTGCCCTGACTGAAGTTCCTGTGCCCACAA
GGTTCTTGTTCATTCTCTTAGGTCCTAAGGGGAAAGCCAAGTCCTACCACGAGATTGGCAGAGCCATTGCCA
CCCTGATGTCTGATGAGGTGTTCCATGACATTGCTTATAAAGCAAAAGACAGGCACGACCTGATTGCTGGTA
TTGATGAGTTCCTAGATGAAGTCATCGTCCTTCCACCTGGGGAATGGGATCCAGCAATTAGGATAGAGCCTC

# FIG. 15A2

CTAAGAGTCTTCCATCCTCTGACAAAAGAAAGAATATGTACTCAGGTGGAGAGAATGTTCAGATGAATGGGG
ATACGCCCCATGATGGAGGTCACGGAGGAGGAGGACATGGGGATTGTGAAGAATTGCAGCGAACTGGACGGT
TCTGTGGTGGACTAATTAAAGACATAAAGAGGAAAGCGCCATTTTTTGCCAGTGATTTTTATGATGCTTTAA
ATATTCAAGCTCTTTCGGCAATTCTCTTCATTTATCTGGCAACTGTAACTAATGCTATCACTTTTGGAGGAC
TGCTTGGGGATGCCACTGACAACATGCAGGGCGTGTTGGAGAGTTTCCTGGGCACTGCTGTCTCTGGAGCCA
TCTTTTGCCTTTTTGCTGGTCAACCACTCACTATTCTGAGCAGCACCGGACCTGTCCTAGTTTTTGAGAGGC
TTCTATTTAATTTCAGCAAGGACAATAATTTTGACTATTTGGAATTTCGCCTTTGGATTGGCCTGTGGTCCG
CCTTCCTATGTCTCATTTTGGTAGCCACTGATGCCAGCTTCTTGGTTCAATACTTCACACGTTTCACGGAGG
AGGGCTTTTCCTCTCTGATTAGCTTCATCTTTATCTATGATGCTTTCAAGAAGATGATCAAGCTTGCAGATT
ACTACCCCATCAACTCCAACTTCAAAGTGGGCTACAACACTCTCTTTTCCTGTACCTGTGTGCCACCTGACC
CAGCTAATATCTCAATATCTAATGACACCACACTGGCCCCAGAGTATTTGCCAACTATGTCTTCTACTGACA
TGTACCATAATACTACCTTTGACTGGGCATTTTTGTCGAAGAAGGAGTGTTCAAAATACGGAGGAAACCTCG
TCGGGAACAACTGTAATTTTGTTCCTGATATCACACTCATGTCTTTTATCCTCTTCTTGGGAACCTACACCT
CTTCCATGGCTCTGAAAAAATTCAAAACTAGTCCTTATTTTCCAACCACAGCAAGAAAACTGATCAGTGATT
TTGCCATTATCTTGTCCATTCTCATCTTTTGTGTAATAGATGCCCTAGTAGGCGTGGACACCCCAAAACTAA
TTGTGCCAAGTGAGTTCAAGCCAACAAGTCCAAACCGAGGTTGGTTCGTTCCACCGTTTGGAGAAAACCCCT
GGTGGGTGTGCCTTGCTGCTGCTATCCCGGCTTTGTTGGTCACTATACTGATTTTCATGGACCAACAAATTA

# FIG. 15A3

CAGCTGTGATTGTAAACAGGAAAGAACATAAACTCAAGAAAGGAGCAGGGTATCACTTGGATCTCTTTTGGG
TGGCCATCCTCATGGTTATATGCTCCCTCATGGCTCTTCCGTGGTATGTAGCTGCTACGGTCATCTCCATTG
CTCACATCGACAGTTTGAAGATGGAGACAGAGACTTCTGCACCTGGAGAACAACCAAAGTTTCTAGGAGTGA
GGGAACAAAGAGTCACTGGAACCCTTGTGTTTATTCTGACTGGTCTGTCAGTCTTTATGGCTCCCATCTTGA
AGTTTATACCCATGCCTGTACTCTATGGTGTGTTCCTGTATATGGGAGTAGCATCCCTTAATGGTGTGCAGT
TCATGGATCGTCTGAAGCTGCTTCTGATGCCTCTGAAGCATCAGCCTGACTTCATCTACCTGCGTCATGTTC
CTCTGCGCAGAGTCCACCTGTTCACTTTCCTGCAGGTGTTGTGTCTGGCCCTGCTTTGGATCCTCAAGTCAA
CGGTGGCTGCTATCATTTTTCCAGTAATGATCTTGGCACTTGTAGCTGTCAGAAAAGGCATGGACTACCTCT
TCTCCCAGCATGACCTCAGCTTCCTGGATGATGTCATTCCAGAAAAGGACAAGAAAAAGAAGGAGGATGAGA
AGAAAAAGAAAAAGAAGAAGGGAAGTCTGGACAGTGACAATGATGATTCTGACTGCCCATACTCAGAAAAAG
TTCCAAGTATTAAAATTCCAATGGACATCATGGAACAGCAACCTTTCCTAAGCGATAGCAAACCTTCTGACA
GAGAAAGATCACCAACATTCCTTGAACGCCACACATCATGCTGATAAAATTCCTTTCCTTCAGTCACTCGGT
ATGCCAAGTCCTCCTAGAACTCCAGTAAAAGTTGTGCCTCAAATTAGAATAGAACTTGAACCTGAAGACAAT
GATTATTTCTGGAGGAGCAAGGGAACAGAAACTACATTGTAACCTGTTTGTCTTTCTTAAAACTGACATTTG
TTGTTAATGTCATTTGTTTTTGTTTGGCTGTTTGTTTATTTTTTAACTTTTATTTCGTCTCAGTTTTTGGTC
ACAGGCCAAATAATACAGCGCTCTCTCTGCTTCTCTCTTGCATAGATACAATCAAGACAATAGTGCACCGTT
CCTTAAAAACAGCATCTGAGGAATCCCCCTTTTGTTCTTAAACTTTCAGATGTGTCCTTTGATAACCAAATT

FIG. 15A4

CTGTCACTCAAGACACAGACACCCACAGACCCTGTCCTTTGCCTCTATTAAGCAGAGGATGGAAGTATTAAG
GATTTTGTAACACCTTTTATGAAAATGTTGAAGGAACTTAAACTTTAGCTTTGGAGCTGTGCTTACTGGCT
TGTCTTTGTCTGGTAGAACAAACCTTGACCTCCAGACAGAGTCCCTTCTCACTTATAGAGCTCTCCAGGACT
GGAAAAAGTGCTGCTATTTTAACTTGCTCTTGCTTGTAAATCCTAATCTTAGAGTTATCAAAAGAAGAAAAA
ACTGAAGGTACTTTACTCCCTATAGAGAAACCATTGCCATCATTGTAGCAAGTGCTGGAATGTCCCTTTTTTT
CCTATGCAACTTTTTTTTAACCCTTTAATGAACTTATCTGTTGAGTACATTGAAGAATATTTTTCTTCCTAGA
TTTTGTTGTTTAAATTATATGGGGCCTAACCTGCCACTTATTTTTGTCAATTTTTAAAACTTTTTTTTAATTA
CTGTAAAGAAAATGAATTTTTTTCCTGCAGCAGGAAACATAATTTTGANTATTCTACCTCTTATTTGTAGCTG
CCAGGCTTTCTGTAAAAATTGTATTGTGATTTTTTACACATACACACACAAATACACACAAT
CTCTAGGGTAAGCCAGAAGGCAAGATCAGATTAAAAACACCATGTTTTCTAAGCATCCATTTTTCCCTTTCTT
TAAAAGAAACTTAACTGTTCTATGAAGGAGATTGAGGGAGAAGAGACAAACTCCTATGTCATGAGAATAACC
GATGTTCTGATAATAGTAGCATCTAGGTACAGATGCTGGTTGTATTACCACGTCAATGTCCTATGCAGTATT
GTTAGACATTTTCTCATTTTGAAATATTTGTGTTTGTGTATGTGCTCTGTGGCCATGGCTGGTGTATATAT
GTGCAATGTTAGAAGGCAAAAGAGTGATGGTAGGCAGAGGGCAAAGTCATTGAATCTCTTATGCCAGTTTTC

FIG. 15B

MEDEAVLDRGASFLKHVCDEEEVEGHHTIYIGVHVPKSYRRRRHKRKTGHKEKKEKERISENYSDKSDIEN
NDESSSSILKPLISPAAERIRFILGEEDDSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEEKVEQGGER
WSKPHVATLSLHSLFELRTCMEKGSIMLDREASSLPQLVEMIVDHQIETGLLKPELKDKVTYTLLRKHRHQT
CKSNLRSLADIGKTVSSASRMFTNPDNGSPAMTHRNLTSFSLNDISDKPEKDQLKNKFMKKLPRDAEASNVL
/GEVDFLDTPFIAFVRLQQAVMLGALTEVPVPTRFLFILLGPKGKAKSYHEIGRAIATLMSDEVFHDIAYKA
QDRHDLIAGIDEFLDEVIVLPPGEWDPAIRIEPPKSLPSSDKRKNMYSGGENVQMNGDTPHDGGHGGGHGD
CEELQRTGRFCGGLIKDIKRKAPFFASDFYDALNIQALSAILFIYLATVTNAITFGGLLGDATDNMQGVLES
FLGTAVSGAIFCLFAGQPLTILSSTGPVLVFERLLFNFSKDNNFDYLEFRLWIGLWSAFLCLILVATDASFL
/QYFTRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINSNFKVGYNTLFSCTCVPPDPANISISNDTTLAPE
KLPTMSSTDMYHNTTFDWAFLSKKECSKYGGNLVGNNCNFVPDITLMSFILFLGTYTSSMALKKFKTSPYFP
FTARKLISDFAIILSILIFCVIDALVGVDTPKLIVPSEFKPTSPNRGWFVPPFGENPWWVCLAAAIPALLVT
ILLIFMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILMVICSLMALPWYVAATVISIAHIDSLKMETETSAP
GEQPKFLGVREQRVTGTLVFILTGLSVFMAPILKFIPMPVLYGVFLYMGVASLNGVQFMDRLKLLLMPLKHQ
PDFIYLRHVPLRRVHLFTFLQVLCLALLWILKSTVAAIIFPVMILALVAVRKGMDYLFSQHDLSFLDDVIPE
KDKKKKEDEKKKKKKGSLDSDNDDSDCPYSEKVPSIKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC

FIG. 16

```
RKNBC5.PRO ----------MSTENV 6
HKNBC5.PRO ----------MSTENV 6
HHNBC5.PRO MEDEAVLDRGASFLKHVCDEEVEGHTIYIGVHVPKSYRRRRRHKRKTG 50

RKNBC5.PRO EGKPNNLGERGRARSSTFLRVFQPMFNHSIFTSAVSPAAERIRFILGEED 56
HKNBC5.PRO EGKPSNLGERGRARSSTFLRVVQPMFNHSIFTSAVSPAAERIRFILGEED 56
HHNBC5.PRO HKEKKEKERISENYSDKSDIENADESSSSILKPLISPAAERIRFILGEED 100

RKNBC5.PRO DSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEE 92
HKNBC5.PRO DSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEE 92
HHNBC5.PRO DSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEE 136
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FIG. 17(1)

cagcctcctccgcgccgcgtcctctggctctctcagtcctcgcggcgccgctgctcctcacccctgagaacgccccctcgcccgcagtcggcgtctcacticcgcggccagctcticcgcgggccag
cgcticcgcacagggtcgccctccgcgtgcccgtctctgccggtcgcggcctctcagagcticccaggcgggcccATGGAGGCAGACGGGGCCGGCGAGCAGAT
GAGACCGCTACTCACGCGGGGTCCCGATGAAGAAGCTGTTGTGGATCTTGGCAAAACTAGCTCAAACCGTGAACACC
AAGTTTGAAAAAGAAGAATTAGAGAGTCATCGAGCTGTATATGTTGGTGTTCATGTACCGTTTAGTAAAGAGAGTCG
TCGGCGTCACAGGCATCGAGGGCACAAACATCACCACCGGAGAAGAAAAGACAAAGACTCAGATAAGGAAGATGG
ACGGGAGTCTCCTTCTTATGACACGCCATCGCAGTTCATCCTTGGGACTGAAGACGATGATGAGGAG
CACATTCCCCACGACCTCTTCACGGAGATGGACGAGCTCTGCTACCGAGACGGGGAAGAGTACGAGTGGAAAGAGA
CAGCCAGGTGGCTGAAGTTCGAAGAGGATGTTGAGGATGGTGGTGACCGATGGAGTAAACCGTATGTGGCCACTCT
TCGAGAAGCTCTTCTGAAGAGACACCATCATCAAAATGAGAAAAGGTTTACGAGTCGGATTCCCCTCGTTCGATCCT
GTCTTTGCACAGTCTCTTTGAGCTGAGGAGTTGTATCCTAAATGGAACAGTCATGCTGGATATGAGAGCAAGCACTC
TTGCAGATATAGGGAAAGGCCTGTCAGCCTCCCGCCATTCTTTGCGAACAGGTCTGTCTGCCTCAAACCTTTCCTTG
TGGATGAAATAGCAGATATGGTGTTAGACAACATGATAGCGTCTCTGGCCAGCTAGATGACTCCATAAGGGAGAATGT
AGAGGAGAATCGCCTTTATCCCTTCTTCTCAGTCATCTTCTTCCTTCTTCAAGAGCTGGCACCCCCTGCAGGCTCAAGG
TGTACCACCCCCAGTACCCACACCCCAGAACAGTCCTCCTTCCAGCCCCAGCTTAAGTCGCCTGACTTCCAGAAGTTC
CCAACAGACTCAGCCTCAGGCCCCAGAAGTACTGGTGTCACCTGACAGGGATGATATTCCCAGAGTAGTAATTCATC
CGCCTGAGGAAGACATAGAAGGCCAAGAGCAGAAGAATGAGGAAAATACTGACTTCACTCCAGGGA
TTTTGGTTTCTCCACAGTCTGCTCCTGGACAGTAGTAAAAGTGGTGAAGGTAACGGAAGTGG

FIG. 17(2)

AGGAAGCAGAGAAAATAGTACTGTTGACTTTAGCAAGGTTGACATGAATTTCATGAGGAAAATTCCTACAGGAGCT
GAGGCATCCAATGTTTTTGGTAGGAGAGGTGGATTTCTTGGAGAGACCTATCATTGCATTTGTGAGACTGGCTCCTGC
AGTTCTCCTCTCAGGGTTGACTGAGGTCCCTGTGCCCACTAGGTTTTTGTTTCTGTTACTGGGCCCAGCAGGAAAGG
CTCCACAGTACCATGAAATTGGCAGATCCATAGCAACTCTAATGACAGATGAGATTTTTCATGATGTAGCTTATAAA
GCAAAAGATCGAAATGACCTCTTATCTGGAATTTTTAGATCAAGTAACTGTTCTTCCTCCAGGAGAGTG
GGATCCTTCCATACGGCATAGAGCCACCAAAAAGTGTTCCTTCTCAGGAGAAAGGAAGATTCCTGCGTTTCCCAATG
GATCTGCTCCAGTGTCTGCTGACCCTCCTAAGGAGGCTGATCACCACGCTGGGCCTGAGCTGCAGAGGACTGGACG
GCTTTTTGGTGGTTTGATACTTGACATCAAAAGGAAAGCACCTTTTTTCTTGAGTGACTTCAAGGATGCATTAAGTCT
GCAGTGCCTGGCCTCGATTCTTTTCCTATACTGTGCCTGTATGTCTCCTGTAATCACTTTTGGAGGGCTGCTTGGAGA
AGCTACAGAAGGCAGAATAAGTGCAATAGAGTCTCTTTTTGGAGCATCATTAACTGGGATTGCCTATTCATTGTTTG
CTGGGCAACCTCTCTAACAATACTGGGGAGCACGGGTCCAGTCCTAGTGTTTGAAAAAATTTTATTTAAATTCTGTAGA
GATTATCACCTATCTATCATTAAGAACCAGTATTGGTCTGTGGACTTCTTTCTTGTGCATTGTGTTGGTCGCA
ACAGATGCCAGCAGCCTTGTTGTTACATTACTCGGTTCACAGAAgAAGCTTTCGCCGCACTCATTGTATCATCTTC
ATCTATGAAGCCTTGGAGAAAACTCTTTCACTTAGGAGAAATATATGCATTTAACATGCACAACAACTTGGATGCATT
GACCAGTTACACATGTGTATGTGCTGAGCCATCTAATCCTAGCAACGAAACTGTAGAGCTGTGGGAgAGGAAGAAC
GTGACAGCAGCCAGTATTTCCTGGGCAAACCTTACCGTGTCTGTGTAAGACCTTCCACGGTATGTTTGTGGGATC
AGCTTGTGGGCCTAACGGGCCTTATGTTCCCGATGTGCTCTTCTGGTGTCGTCTTGTTTTTCACAACGTTCTTTCT

FIG. 17(3)

GTCTTCATTCCTCAAGCAGTTTAAGACCAAGAGATATTTCCTACTAAGGTGCGATCAACAATCAGTGACTTTGCTGT
ATTTCTCACAATAGTAATAATGGTTGCAATTGACTACCTTGTAGGAATTCCATCTCCTAAACTTCATGTTCCTGAAAA
GTTTGAGCCTACTGATCCAAGCAGGGGCTGGATCATAAGCCCTTTGGGAGATAACCCTTGGCGGACCTTACTAATTG
CAGCTGTTCCAGCTCTCCTTTGTACCATTCTCATCTTCATGGACCAGCAGATCACAGCTGTGATCATCAACAGGAAA
GAACACAAACTGAAGAAAGGAGGCTGGCTATCACCTGGATTTGCTCATGGTTGCTGTCATGTTGGGAGTCTGCTCCAT
CATGGGCCTGCCGTGGTTTGTGGCTGCAACAGTGTTGTCTATAAGTCATGTCAAGCTTAAAAGTAGAGTCTGAAT
GTTCTGCTCCAGGGGAACAGCCCAAGTTCCTCGTGAATTCGTGAACAGCGGGGTTACAGGGCTGATGATTTTTATCCTG
ATGGGCCTCTCTCTGTGTTCATGACTTCAGTATTAAAGTTTATTCCGATGCCAGTTCTATATGGTGTTTTCCTTTATATG
GGAGTTTCTTCTCTGAAAGGAATTCAGTTTTTTTGACCGTATCAAATTATTTGGAATGCCTGCCAAGCACCAGCCAGA
CCTGATCTACCTCCGCTATGTCCCTCTCTGGAAGGTGCACGTGTTCACGGTCGTCCAGCTGACCTGCCTGGTTCTGC
TCTGGGTGATCAAAGCCTCTCTGCTGCTGCAGTAGTTTTTCCCATGATGGTTCTTGCATTAGTCTTTTGTGCGCAAGCTCA
TGGATCTGTGCTTCACAAAGAGAGAACTCAGTTGGCTTGATGACCTCATGCCAGAAAGTAAGAAAAAGAAAGAAGA
TGACAAGAAGAAGAAGGAGAAGGAGGAAGCTGAGCGGATGCTTCAGGGTGACGGGGATACTGTGCACCTCCCATT
CGAAAGGGGGAGTCTCCTACAAATTCCAGTTAAGACCCTAAAATATAGTATTGACCCTTCAGTTGTTAACATATCAG
ATGAAATGGCCAAAACTGCCCAGTGGAAGGCACTTTCCATGAATACTGAGAAATGCCAAAGTAACCAGACCTAACAT
GAGCCCTGAAAAGCCTGTGAGTGTGACAATAAATTTCGAAGATGAACCATCAAAAAAAATACATGGATGCTGAAACT
TCATTGtaaagtcaaaccaaaaggaatatatagagatatatgcctatctgtgtatgcacaaacacacacacacacactactatgc

FIG. 18(1)

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jactagaaagaaactgctagATGGCTGTAACACAGTTCATCCATTTCCGTGAAGAGATCATGGGGAATATGT
:CTTCATCATCATCTTCAGTACCAAGGATAAACTGTGTTACAGAGATGGAGAAGAATATGAATGGAAAGAAA
:TGCTAGATGGCTGAAATTTGAAGAGGATGTTGAAGATGGCGGTGACCGATGGAGTAAACCTTATGTGGCAA
:TCTCTCTTTGCACAGTCTTTTTGAACTAAGGAGTTGCATCCTCAATGGAACAGTCATGCTGGATATGAGAG
:AAGCACTCTAGATGAAATAGCAGATATGGTATTAGACAACATGATAGCTTCTGGCCAATTAGACGAGTCCA
TACGAGAGAATGTCAGAGAAGCTCTTCTGAAGAGACATCATCATCAGAATGAGAAAAGATTCACCAGTCGGA
TTCCTCTTGTTCGATCTTTTGCAGATATAGGCAAGAAACATTCTGACCCTCACTTGCTTGAAAGGAATGGTA
TTTTGGCCTCTCCCCAGTCTGCTCCTGGAAACTTGGACAATAGTAAAAGTGGAGAAATTAAAGGTAATGGAA
GTGGTGGAAGCAGAGAAAATAGTACTGTTGACTTCAGCAAGGTTGATATGAATTTCATGAGAAAAATTCCTA
CGGGTGCTGAGGCATCCAACGTCCTGGTGGGCGAAGTAGACTTTTTGGAAAGGCCAATAATTGCATTTGTGA
GACTGGCTCCTGCTGTCCTCCTTACAGGGTTGACTGAGGTCCCTGTTCCAACCAGGTTTTTGTTTTTGTTAT
TGGGTCCAGCGGGCAAGGCACCACAGTACCATGAAATTGGACGATCAATAGCCACTCTCATGACAGATGAGA
TTTTCCATGATGTAGCTTATAAAGCAAAAGACAGAAATGACCTCTTATCTGGAATTGATGAATTTTTAGATC
AAGTAACTGTCCTACCTCCAGGAGAGTGGGATCCTTCTATACGCATAGAACCACCAAAAAGTGTCCCTTCTC
AGGAAAAGAGAAAGATTCCTGTGTTTCACAATGGATCTACCCCCACACTGGGTGAGACTCCTAAAGAGGCCG
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FIG. 18(2)

CTCATCATGCTGGGCCTGAGCTACAGAGGACTGGACGGCTTTTTGGTGGTTTGATACTTGACATCAAAAGGA
AAGCACCTTTTTTTCTTGAGTGACTTCAAGGATGCATTAAGCCTGCAGTGCCTGGCCTCGATTCTTTTCCTAT
ACTGTGCCTGTATGTCTCCTGTAATCACTTTTGGAGGGCTGCTTGGAGAAGCTACAGAAGGCAGAATAAGTG
CAATAGAGTCTCTTTTTGGAGCATCATTAACTGGGATTGCCTATTCATTGTTTGCTGGGCAACCTCTAACAA
TATTGGGGAGCACAGGTCCAGTTCTAGTGTTTGAAAAAATTTTATATAAATTCTGCAGAGATTATCAACTTT
CTTATCTGTCTTTAAGAACCAGTATTAGTCTGTGGACTTCTTTTTTGTGCATTGTTTTGGTTGCAACAGATG
CAAGCAGCCTTGTGTGTTATATTACTCGATTTACAGAAGAGGCTTTTGCAGCCCTTATTTGCATCATATTCA
TCTACGAGGCTTTGGAGAAGCTCTTTGATTTAGGAGAAACATATGCATTTAATATGCACAACAACTTAGATA
AACTGACCAGCTACTCATGTGTATGTACTGAACCTCCAAACCCCAGCAATGAAACTCTAGCACAATGGAAGA
AAGATAATATAACAGCACACAATATTTCCTGGAGAAATCTTACTGTTTCTGAATGTAAAAAACTTCGTGGTG
TATTCTTGGGGTCAGCTTGTGGTCATCATGGACCTTATATTCCAGATGTGCTCTTTTGGTGTGTCATCTTGT
TTTTCACAACATTTTTTCTGTCTTCATTCCTCAAGCAATTTAAGACCAAGCGTTACTTTCCTACCAAGGTGC
GATCGACAATCAGTGATTTTGCTGTATTTCTCACAATAGTAATAATGGTTACAATTGACTACCTTGTAGGAG
TTCCATCTCCTAAACTTCATGTTCCTGAAAAATTTGAGCCTACTCATCCAGAGAGAGGGTGGATCATAAGCC
CACTGGGAGATAATCCTTGGTGGACCTTATTAATAGCTGCTATTCCTGCTTTGCTTTGTACCATTCTCATCT

FIG. 18(3)

TTATGGATCAACAAATCACAGCTGTAATTATAAACAGAAAGGAACACAAATTGAAGAAAGGAGCTGGCTATC
ACCTTGATTTTGCTCATGGTTGGCGTTATGTTGGGAGTTTGCTCTGTCATGGGACTTCCATGGTTTGTGGCTG
CAACAGTGTTGTCAATAAGTCATGTCAACAGCTTTAAAAGTTGAATCTGAATGTTCTGCTCCAGGGGAACAAC
CCAAGTTTTTTGGGAATTCGTGAACAGCGGGTTACAGGGCTAATGATTTTTATTCTAATGGGCCTCTCTGTGT
TCATGACTTCAGTCCTAAAGTTTATTCCAATGCCTGTTCTGTATGGTGTTTTCCTTTATATGGGAGTTTCCT
CATTAAAAGGAATCCAGTTATTTGACCGTATAAAATTATTTGGAATGCCTGCTAAGCATCAGCCTGATTTGA
TATACCTCCGTTATGTGCCGCTCTGGAAGGTCCATATTTTCACAGTCATTCAGCTTACTTGTTTGGTCCTTT
TATGGGTGATAAAAGTTTCAGCTGCTGCAGTGGTTTTTCCCATGATGGTTCTTGCATTAGTGTTTGTGCGCA
AACTCATGGACCTGTGTTTCACGAAGAGAACTTAGTTGGCTTGATGATCTTATGCCAGAAAGTAAGAAAA
AGAAAGAAGATGACAAAAAAGAGAAAGAGGAAGCTGAACGGATGCTTCAAGACGATGATACTG
TGCACCTTCCATTTGAAGGGGGAAGTCTCTTGCAAATTCCAGTCAAGGCCCTAAAATATAGTCCTGATAAAC
CTGTGAGTGTGAAAATAAGTTTTGAAGATGAACCAAGAAAGAAATACGTGGATGCTGAAACTTCATTAtaga
attgaaccaagaggcattatatacatatagataatgtgtgcgtatcatgtcactatataataa
gaatattgtatgtcatgctgtttaagttgtgactaccgggtttttaaagtagtgtctggagtttgtatgagc
ancgtggagactatgtatttaa

FIG. 19A1

ACTAGAAAGAAACTGCTAGATGGCTGTAACACAGTTCATCCATTTCCGTGAAGAGATCATGGGGAATATGTT
CTTCATCATCATCTTCAGTACCAAGGATAAACTGTGTTACAGAGATGGAGAAGAATATGAATGGAAAGAAAC
TGCTAGATGGCTGAAATTTGAAGAGGATGTTGAAGATGGCGGTGACCGATGGAGTAAACCTTATGTGGCAAC
TCTCTCTTTGCACAGTCTTTTTGAACTAAGGAGAGTTGCATCCTCAATGGAACAGTCATGCTGGATATGAGAGC
AAGCACACTCTAGATGAAATAGCAGATATGGTATTAGACAACATGATAGCTTCTGGCCAATTAGACGAGTCCAT
ACGAGAGAATGTCAGAGAAGCTCTTCTGAAGAGACATCATCATCAGAATGAGAAAAGATTCACCAGTCGGAT
TCCTCTTGTTCGATCTTTTTGCAGATATAGGCAAGAAACATTCTGACCCTCACTTGCTTGAAAGGAATGGTAT
TTTGGCCTCTCCCCAGTCTGCTCCTGGAAACTTGGACAATAGTAAAAGTGGAGAAATTAAAGGTAATGGAAG
TGGTGGAAGCAGAGAAAATAGTACTGTTGACTTCAGCAAGGTTGATATGAATTTCATGAGAAAAATTCCTAC
GGGTGCTGAGGCATCCAACGTCCTGGTGGGCGAAGTAGACTTTTTGGAAAGGCCAATAATTGCATTTGTGAG
ACTGGCTCCTGCTGTCCTCCTTACAGGGTTGACTGAGGTCCCTGTTCCAACCAGGTTTTTTGTTTTATT
GGGTCCAGCGGGCAAGGCACCACACAGTACCATGAAATTGGACGATCAATAGCCACTCTCATGACAGATGAGAT
TTTCCATGATGTAGCTTATAAAGCAAAAGACAGAAATGACCTCTTATCTGGAATTGATGAATTTTTAGATCA
AGTAACTGTGTCCTACCTCCAGGAGAGTGGGATCCTTCTATACGCATAGAACCACCAAAAAGTGTCCCTTCTCA
GGAAAAGAGAAAGATTCCTGTGTTTCACAATGGATCTACCCCCACACTGGGTGAGACTCCTAAAGAGGCCGC
TCATCATGCTGGGCCTGAGCTACAGAGGACTGGACGGCTTTTTTGGTGGTTTGATACTTGACATCAAAAGGAA
AGCACCTTTTTTTCTTGAGTGACTTCAAGGATGCATTAAGCCTGCAGTGCCTGGCCTCGATTCTTTTCCTATA
CTGTGCCTGTATGTCTCCTGTAATCACTTTTGGAGAGGGCTGCTTGGAGAAGGCTACAGAAGGCAGAATAAGTGC
AATAGAGTCTCTTTTTGGAGCATCATTAACTGGGATTGCCTATTCATTGTTTGCTGGGCAACCTCTAACAAT
ATTGGGGAGCACAGGTCCAGTTCTAGTGTTTGAAAAAATTTTATATAAATTCTGCAGAGATTATCAACTTTC

# FIG. 19A2



ΓTATCTGTCTTTAAGAACCAGTATTRGTCTGTGGACTTCTTTTTTGTGCATTGTTTTGGTTGCAACAGATGC
ΛAGCAGCCTTGTGTGTTATATTACTCGATTTACAGAAGAGGCTTTTGCAGCCCTTATTTGCATCATATTCAT
ΣTACGAGGCTTTGGAGAAGCTCTTTGATTTAGGAGAAACATATGCATTTAATATGCACAACAACTTAGATAA
ΛCTGACCAGCTACTCATGTGTATGTACTGAACCTCCAAACCCCAGCAATGAAACTCTAGCACAATGGAAGAA
ΛGATAATATAACAGCACACAATATTTCCTGGAGAAATCTTACTGTTTCTGAATGTAAAAAACTTCGTGGTGT
ΛTTCTTGGGGTCAGCTTGTGGTCATCATGGACCTTATATTCCAGATGTGCTCTTTTGGTGTGTCATCTTGTT
ΓTTCACAACATTTTTTCTGTCTTCATTCCTCAAGCAATTTAAGACCAAGCGTTACTTTCCTACCAAGGTGCG
ΛTCGACAATCAGTGATTTTGCTGTATTTCTCACAATAGTAATAATGGTTACAATTGACTACCTTGTAGGAGT
ΓCCATCTCCTAAACTTCATGTTCCTGAAAAATTTGAGCCTACTCATCCAGAGAGAGGGTGGATCATAAGCCC
ΛCTGGGAGATAATCCTTGGTGGACCTTATTAATAGCTGCTATTCCTGCTTTGCTTTGTACCATTCTCATCTT
ΓATGGATCAACAAATCACAGCTGTAATTATAAACAGAAAGGAACACAAATTGAAGAAAGGAGCTGGCTATCA
CCTTGATTTGCTCATGGTTGGCGTTATGTTGGGAGTTTGCTCTGTCATGGGACTTCCATGGTTTGTGGCTGC
AACAGTGTTGTCAATAAGTCATGTCAACAGCTTAAAAGTTGAATCTGAATGTTCTGCTCCAGGGGAACAACC
CAAGTTTTTGGGAATTCGTGAACAGCGGGTTACAGGGCTAATGATTTTTATTCTAATGGGCCTCTCTGTGTT
CATGACTTCAGTCCTAAAGTTTATTCCAATGCCTGTTCTGTATGGTGTTTTCCTTTATATGGGAGTTTCCTC
ATTAAAAGGAATCCAGTTATTTGACCGTATAAAATTATTTGGAATGCCTGCTAAGCATCAGCCTGATTTGAT
ATACCTCCGTTATGTGCCGCTCTGGAAGGTCCATATTTTCACAGTCATTCAGCTTACTTGTTTGGTCCTTTT
ATGGGTGATAAAAGTTTCAGCTGCTGCAGTGGTTTTTCCCATGATGGTTCTTGCATTAGTGTTTGTGCGCAA
ACTCATGGACCTGTGTTTCACGAAGAGAGAACTTAGTTGGCTTGATGATCTTATGCCAGAAAGTAAGAAAAA
GAAAGAAGATGACAAAAAGAAAAAAGAGAAAGAGGAAGCTGAACGGATGCTTCAAGACGATGATGATACTGT

# FIG. 19A3

GCACCTTCCATTTGAAGGGGGAAGTCTCTTGCAAATTCCAGTCAAGGCCCTAAAATATAGTCCTGATAAACC
TGTGAGTGTGAAAATAAGTTTTGAAGATGAACCAAGAAAGAAATACGTGGATGCTGAAACTTCATTATAGAA
TTGAACCAAGAGGCATTATACATATAGATATATACATATGTAATGTGTGCGTATCATGTCACTATATATAAG
AATATTGTATGTCATGCTGTTTATGTGTGACTACCGGGTTTTTAAAAGTAGTGTCTGGAGTTTGTAATGAGC
ACCGTGGAGACTATGTATTTAATGAAATGCTCTCTTTGAAGTGAGGTACATGGTTCTTAACTATTCAAATAT
TTATTCTGTTAGAAAAAAAAATTTTCTGTTTTGCAATAGAAGGATGTGGAGAAATGCTTTCAGTCTACTTTT
CTTAAATCTCTGTTCATCAGTGGCAATTCGTAAAAACCTTAAGTGATACTTTGTTTATATGTTTATAATTTT
TAGGTGTTTCCTGAAATTTTCACATATTATTTCACTTTTGTTAGTGCTTTATGGGAAGAATAGGGAGTCTAT
ACCAGTGCTGTGGGAAAAATGGTAACATTTCAGGGCTTCTCTATTTGTGTTTCATTTCTGTAGATGTCCATC
GTGTTTCACTAACTGGCGTTTTCTTAGCCATAGAGATGACTGTAGAACAATAGAAACTTTAATAATGATAGT
TTTTAACTTTTATGTTTAAATTTTTTTTAAATCTTAAAACCTTCATATCTAGGTATCCATTGTGACAGACAA
GTAAAATTGCAGGTGATTTGATAATTAAGCACCCATACCATTTATAACTTCTGAATTTAAAAAGTTATACAA
TGCCAGTTTGCAATAGTTGATTTTGATGCCTTTGTAGAATATTTTTTCTGAATCCTTATGCTCTTTTAAAGC
CAATGATTCCCACTCTGTTCTCTGCCTTGTCTCTCTTTGTCTTAAAATGCTTTAGTTTCCATCAGGTTCAAG
TTCTTGACTATTATTCTCTTATAAGTAGTAGGCGTAAATAATCAGGAGTTAGAATTCTCTCAGAAGGGTCTA
TGATCAGTATTACTTTATTAAGAATTACCTTTCATTTTCTCTTTATGTTATTCTTTCACTTTTGTAGATTAC
ATTTAATAGCTTGTTACCTGTGATTTTATTTTAAAATATTTATTTTGATATGATGCTTAAATATTATATAAA
CATTTGGAAAAGTAACAAATAGAGTAAATTGTTAATGTAAATAGTTGGTGCTTACTTTCATTTATGTTTATT
ATCTTAAAGCAATTGATAGATTTTACATCTTTGATATAAAGCACTGCCATATTTATATTTTAAAAGGAAATT
AGACATTTTATATGTAGCTCAGATTAATGACATTTTTATTTTGTGTATTAGTTTTTGCTTTTCTGAGCTTTT
TAAAGTCTAACAAATCTTTTTAGTCATCTTTTATATACTTTTAGTTCCACATGAAATAAATGTTGTTAAGCC
TGTAAAAAAAAAAAAAAAAAAAAA



WO 98/53067 PCT/US98/10297

# FIG. 19B

MAVTQFIHFREEIMGNMFFIIIFSTKDKLCYRDGEEYEWKETARWLKFEEDVEDGGDRWSKPYVATLSLHSL
FELRSCILNGTVMLDMRASTLDEIADMVLDNMIASGQLDESIRENVREALLKRHHHQNEKRFTSRIPLVRSF
ADIGKKHSDPHLLERNGILASPQSAPGNLDNSKSGEIKGNGSGGSRENSTVDFSKVDMNFMRKIPTGAEASN
VLVGEVDFLERPIIAFVRLAPAVLLTGLTEVPVPTRFLFLLLGPAGKAPQYHEIGRSIATLMTDEIFHDVAY
KAKDRNDLLSGIDEFLDQVTVLPPGEWDPSIRIEPPKSVPSQEKRKIPVFHNGSTPTLGETPKEAAHHAGPE
LQRTGRLFGGLILDIKRKAPFFLSDFKDALSLQCLASILFLYCACMSPVITFGGLLGEATEGRISAIESLFG
ASLTGIAYSLFAGQPLTILGSTGPVLVFEKILYKFCRDYQLSYLSLRTSIXLWTSFLCIVLVATDASSLVCY
ITRFTEEAFAALICIIFIYEALEKLFDLGETYAFNMHNNLDKLTSYSCVCTEPPNPSNETLAQWKKDNITAH
NISWRNLTVSECKKLRGVFLGSACGHHGPYIPDVLFWCVILFFTTFFLSSFLKQFKTKRYFPTKVRSTISDF
AVFLTIVIMVTIDYLVGVPSPKLHVPEKFEPTHPERGWIISPLGDNPWWTLLIAAIPALLCTILIFMDQQIT
AVIINRKEHKLKKGAGYHLDLLMVGVMLGVCSVMGLPWFVAATVLSISHVNSLKVESECSAPGEQPKFLGIR
EQRVTGLMIFILMGLSVFMTSVLKFIPMPVLYGVFLYMGVSSLKGIQLFDRIKLFGMPAKHQPDLIYLRYVP
LWKVHIFTVIQLTCLVLLWVIKVSAAAVVFPMMVLALVFVRKLMDLCFTKRELSWLDDLMPESKKKKEDDKK
KKEKEEAERMLQDDDDTVHLPFEGGSLLQIPVKALKYSPDKPVSVKISFEDEPRKKYVDAETSL

FIG. 20A1

TGATGGTTGACCGTTGGCTCCGGGGGTGGGGGTCGCCGTTCGAGTGATCTGCTCAGACCCGACCAGAGGGCGC
GGGCTGCTGATGCTTGGCTTGGAGCCCGTGGGGGAGACCTAGTTCGGCTCCGCCATGCCGGCCGCCGGGAGT
AACGAGCCGGACGGCGTCCTCAGCTATCAGAGACCAGATGAAGAAGCTGTGGTGGATCAGGGTGGGACCAGT
ACAATTCTCAACATTCACTATGAAAAAGAAGAGCTGGAAGGTCACAGAACTCTGTATGTGGGAGTTCGGATG
CCGCTTGGCCGGCAGAGCCATCGGCATCACCGCACTCATGGCCAGAAGCACCGGAGACGAGGGCGGGGCAAA
GGAGCCAGCCAGGGGGAGGAAGGCCTGGCCCACGACACACCATCTCAGCGTGTTCAGTTCATT
CTTGGCACCGAGGAAGATGAAGAGCATGTGCCTCATGAGCTGTTTACAGAGCTGGATGAGATCTGTATGAAA
GAGGGAGAAGATGCTGAGTGGAAGGAAACAGCCAGGTGGCTGAAGTTTGAAGAAGATGTTGAAGATGGGGGA
GAACGCTGGAGCAAGCCTTATGTGGCAACCCTTTCATTGCACAGCCTGTTTGAGCTAAGGAGCTGCCTTATT
AATGGAACAGTCCTCCTGGATATGCATAGCATAGAAGAAATTTCAGACCTGATCCTGGATCAGCAA
GAACTGTCCAGTGACCTGAATGACAGCATGAGGGTTAAAGTGCGGGAAGCCCTTCTCAAAAAGCATCATCAT
CAGAATGAAAAGAAGAGAAACAACCTCATTCCCATTGTTCGCTCCTTTGCTGAGGTTGGCAAGAAGCAGTCT
GATCCTCATTTGATGGATAAACATGGTCAAACCGTGTCTCCTCAGTCTGTTCCAACTACAAATCTTGAAGTA
AAAAATGGAGTGAATTGTGAACATAGTCCTGTGGATTTAAGCAAGGTAGACCTTCATTTCATGAAAAAAATT
CCTACTGGGGCCGAGGCCTCCAATGTCCTGGTTGGAGAGGTGGATATTTTTGGACCGTCCCATTGTTGCCTTT
GTGAGGCTGTCTCCAGCTGTTCTTCTCTCAGGCCTAACAGAAGTGCCAATCCCAACAAGATTTTTGTTTATC
TTATTGGGTCCAGTAGGGAAAGGTCAGCAGTACCATGAGATTGGCAGATCCATGGCCACCATCATGACAGAT
GAGATTTTTCATGACGTAGCATATAAGGCAAAAGAGCGAGATGATCTCCTGGCGGGGATTGATGAGTTCCTA
GACCAGGTGACGGTGCTCCCTCCAGGAGAGTGGGATCCCTCCATTAGAATTGAGCCACCCAAAAATGTCCCT

FIG. 2042

TCCCAGGAGAAAAGGAAAATGCCTGGAGTTCCAAATGTTTGCCACATAGAACAGGAACCACATGGG
GGTCACAGTGGGCCAGAACTTCAGCGCACTGGGCGGCTATTTGGGGGCTTGGTGCTGGACATCAAGCGGAAG
GCCCCCTGGTACTGGAGCGACTACCGAGATGCACACTCAGCTTACAGTGTTTGGCTTCCTTTCTGTTCCTGTAC
TGTGCCTGCATGTCACCTGTCATCACCTTTGGGGGACTGCTTGGAGAAGCCACTGAGGGACGCATAAGTGCA
ATTGAATCCTTGTTTGGAGCTTCCATGACTGGGATTGCTTATTCCTTGTTTGCGGGACAGGCTCTCACCATC
CTGGGAAGTACTGGACCAGTGCTTGTGTTTGAAAAGATTTTGTTCAAATTCTGCAAAGACTATGCTCTTTCA
TACCTCTCCCTGCGAGCTTGTATTGGACTGTGGACCGCTTTCCTGTGTATTGTCCTTGTGGCAACTGATGCC
AGTTCCCTTGTCTGCTACATTACCCGTTTCACTGAAGAAGCATTTGCCTCCCTAATTTGCATTACTTTTCATC
TATGAAGCAATAGAAAAACTGATTCACCTGGCAGAGACCTACCCCATCCCACATGCACAGCCAGCTGGACCAC
CTTAGCCTCTATTACTGCAGGTGTACTCTGCCAGAGAATCCAATCACACCCTCCAGTACTGGAAGGAC
CACAACATCGTGACAGCAGAAGTCCACTGGGCTAACCTGACTGTCAGTGAATGCCAGGAGATGCATGGAGAG
TTCATGGGATCTGCGTGCGGCCATCATGGACCCCTACACTCCCTGATGTCCTCTTTTGGTCCTGTATTCTCTTT
TTCACCACCTTCATCCTCTCAAGCACCTTAAAGACGAGCCGTTATTTCCCAACCAGAGTACGC
TCCATGGTGAGTGACTTTGCTGTTTCCTCACTATCTTCACAATGGTGATTATTTTTGATTGGAGTC
CCATCACCAAAGCTTCAAGTTCCCAGTGTGTTCAAGCCAACAAGGGATGATCGCGGGATGGATTATTAATCCC
ATTGGCCCCCAATCCCCTGGTGGACTGTGATAGCTGCAATTATCCCAGCTCTTCTCTGTACTATCTTGATATTC
ATGGNTCAGCAGATCACACAGCCGTCATTANTAACAGGAAGGAAGCATAAGCTCAAGAAAGGCTGTGGCTACCAC
CTGGACCTACTGATGGTGGCCATCATGCTGGGTGTCTGCTCCATCATGGGCCCTGCCCTGGTTTGTAGCTGCA
ACTGTC

FIG. 20B

MPAAGSNEPDGVLSYQRPDEEAVVDQGGTSTILNIHYEKEELEGHRTLYVGVRMPLGRQS
HRHHRTHGQKHRRRGRGKGASQGEEGLEALAHDTPSQRVQFILGTEEDEEHVPHELFTEL
DEICMKEGEDAEWKETARWLKFEEDVEDGGERWSKPYVATLSLHSLFELRSCLINGTVLL
DMHANSIEEISDLILDQQELSSDLNDSMRVKVREALLKKHHHQNEKKRNNLIPIVRSFAE
VGKKQSDPHLMDKHGQTVSPQSVPTTNLEVKNGVNCEHSPVDLSKVDLHFMKKIPTGAEA
SNVLVGEVDILDRPIVAFVRLSPAVLLSGLTEVPIPTRFLFILLGPVGKGQQYHEIGRSM
ATIMTDEIFHDVAYKAKERDDLLAGIDEFLDQVTVLPPGEWDPSIRIEPPKNVPSQEKRK
MPGVPNGNVCHIEQEPHGGHSGPELQRTGRLFGGLVLDIKRKAPWYWSDYRDALSLQCLA
SFLFLYCACMSPVITFGGLLGEATEGRISAIESLFGASMTGIAYSLFAGQALTILGSTGP
VLVFEKILFKFCKDYALSYLSLRACIGLWTAFLCIVLVATDASSLVCYITRFTEEAFASL
ICIIFIYEAIEKLIHLAETYPIHMHSQLDHLSLYYCRCTLPENPNNHTLQYWKDHNIVTA
EVHWANLTVSECQEMHGEFMGSACGHHGPYTPDVLFWSCILFFTTFILSSTLKTFKTSRY
FPTRVRSMVSDFAVFLTIFTMVIIDFLIGVPSPKLQVPSVFKPTRDDRGWIINPIGPNPW
WTVIAAIIPALLCTILIFM

FIG. 21

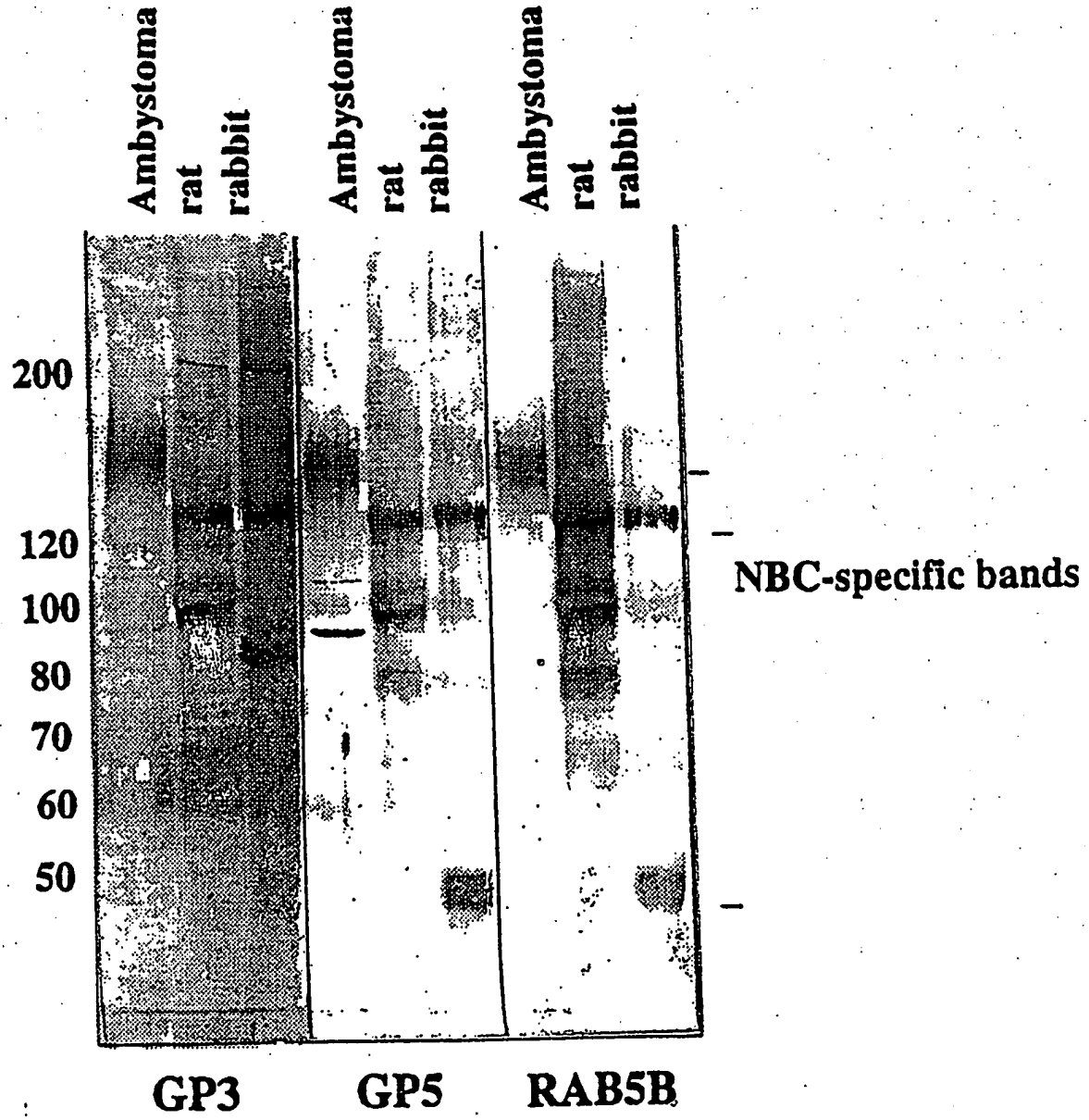
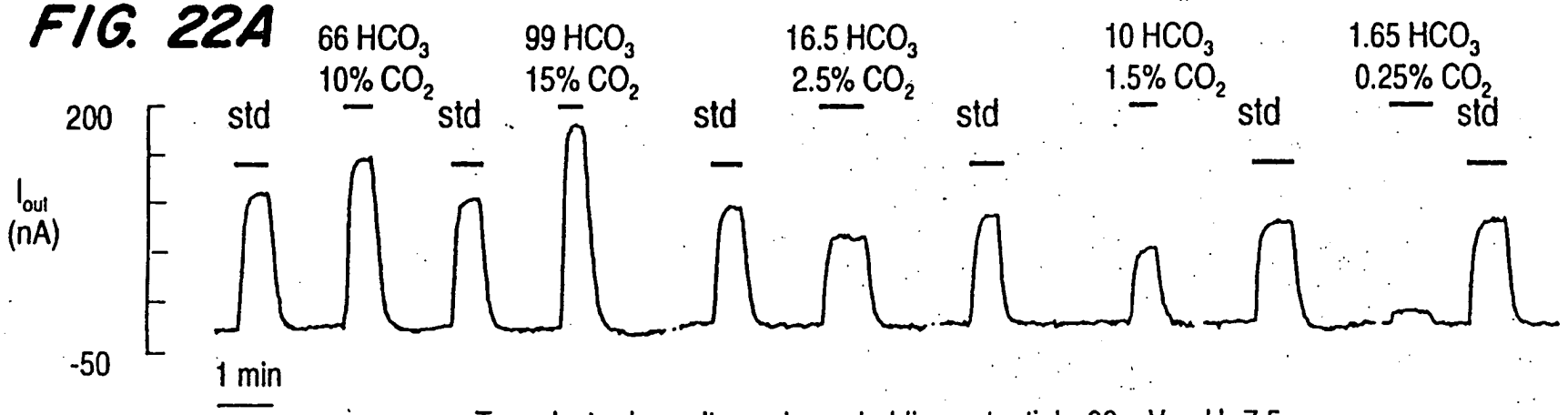

Ambystoma
rat
rabbit
Ambystoma
rat
rabbit
Ambystoma
rat
rabbit
200
120
100
80
70
60
50
NBC-specific bands
GP3
GP5
RAB5B

FIG. 22A




Two-electrodes voltage clamp: holding potential - 60 mV; $pH_o$ 7.5

FIG. 22B

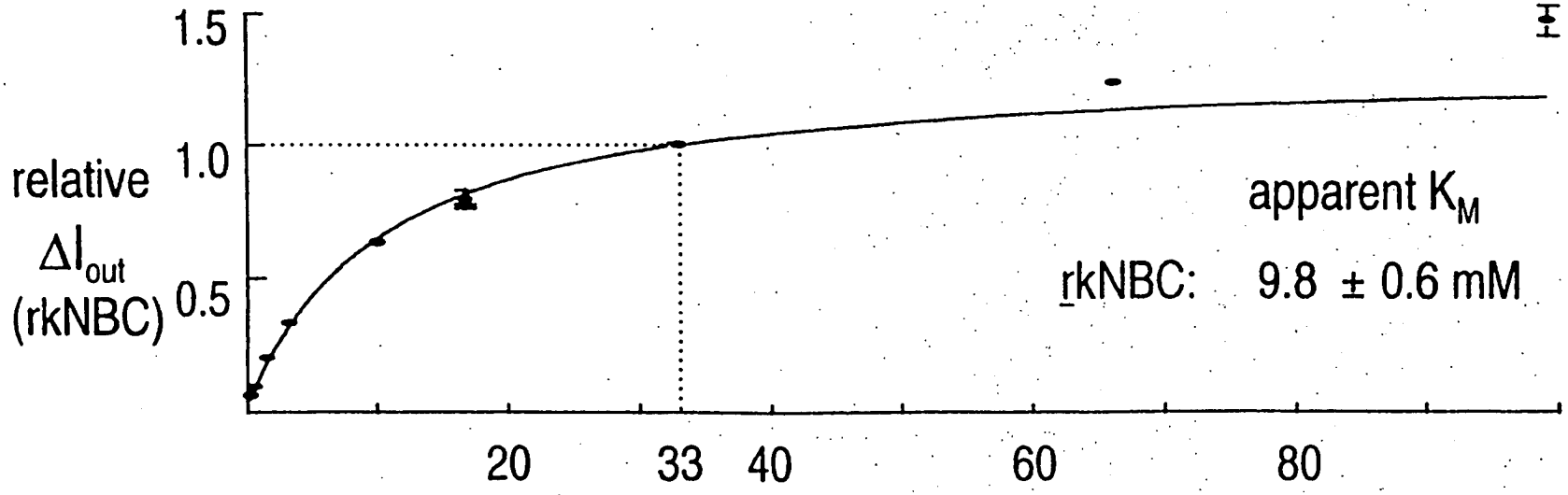


FIG. 23A

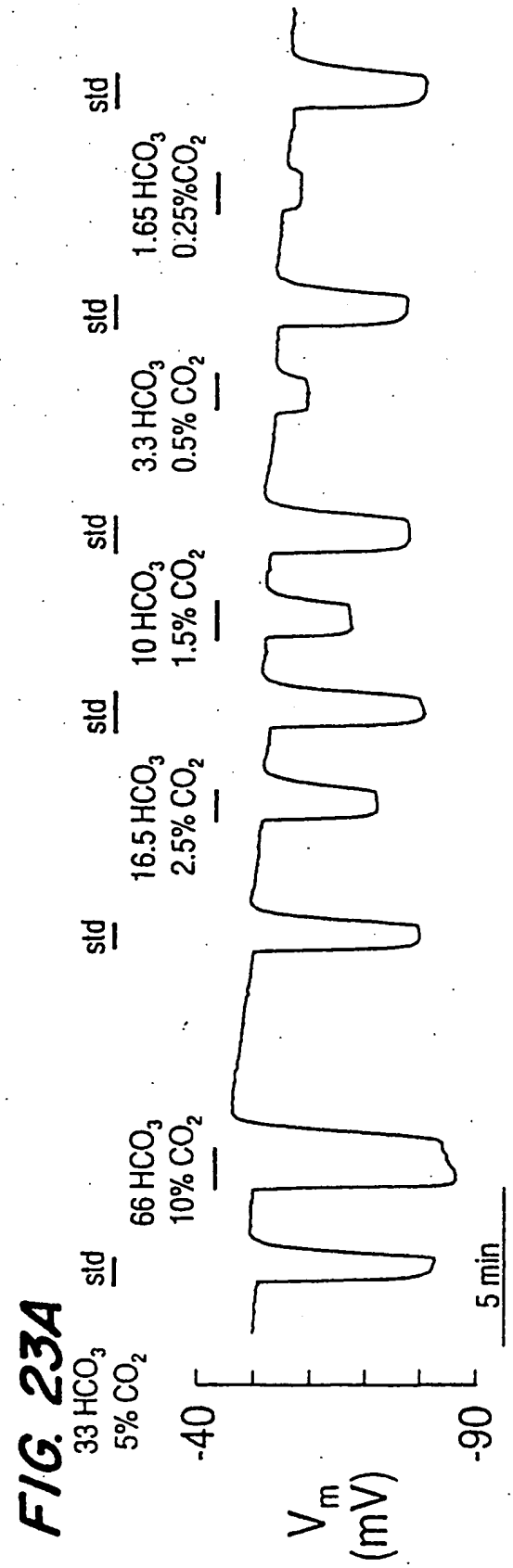



FIG. 23B

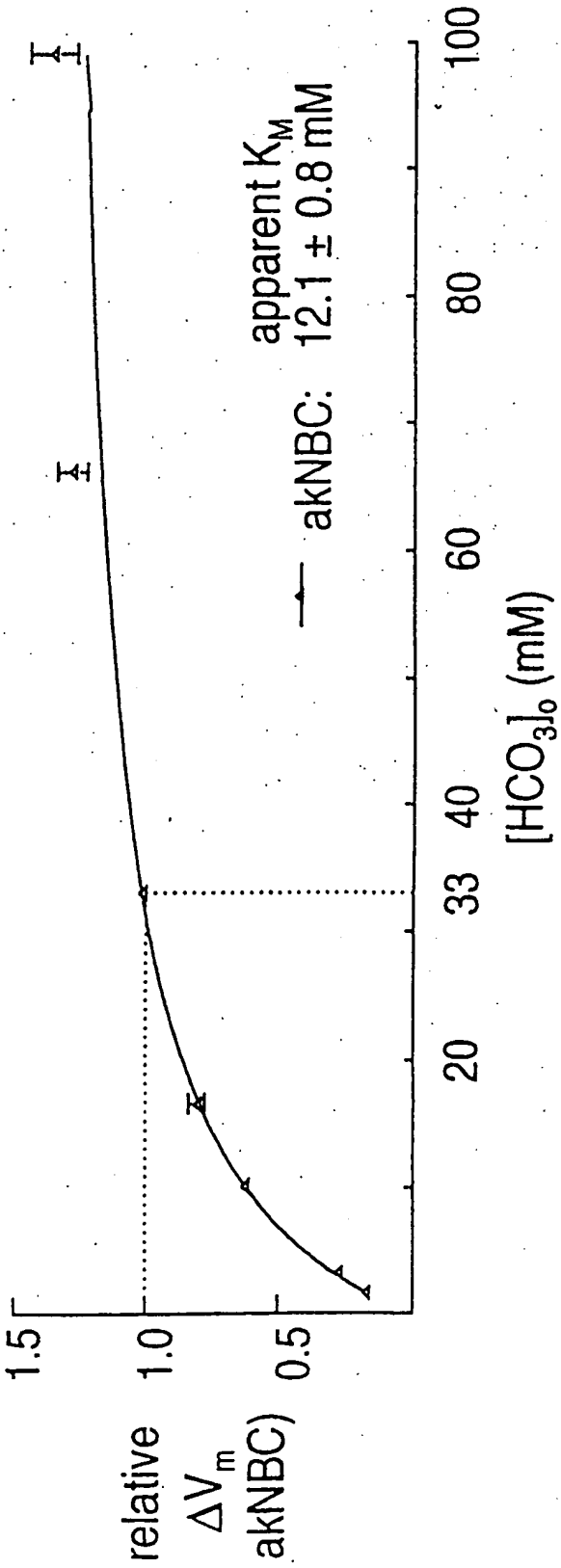


FIG. 24A

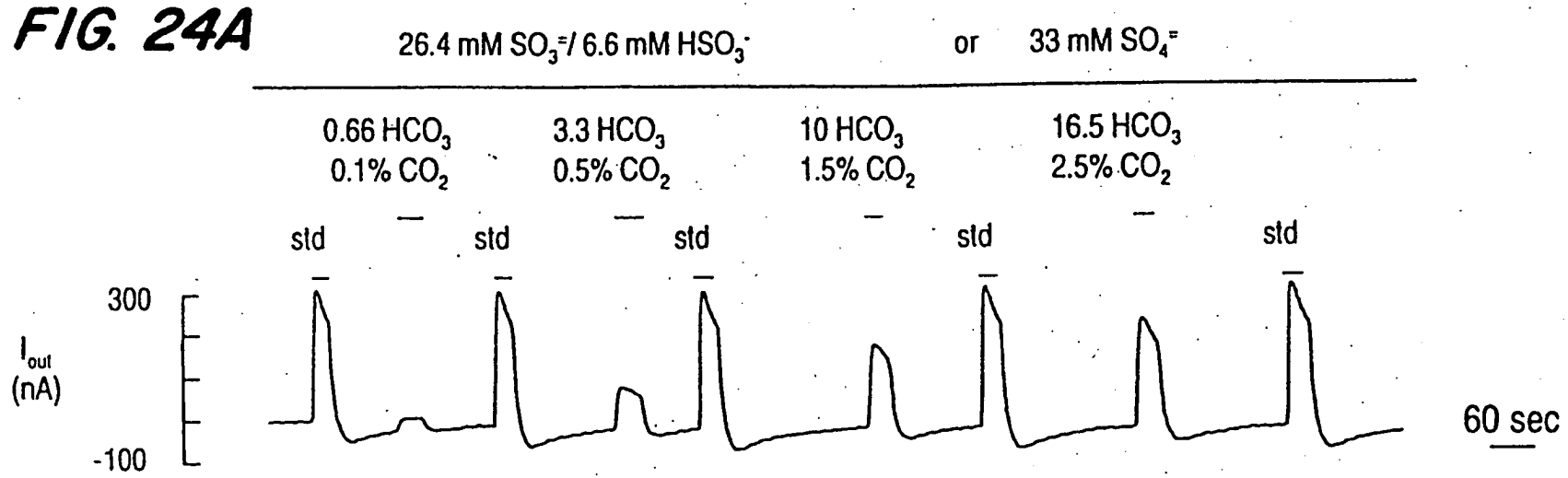
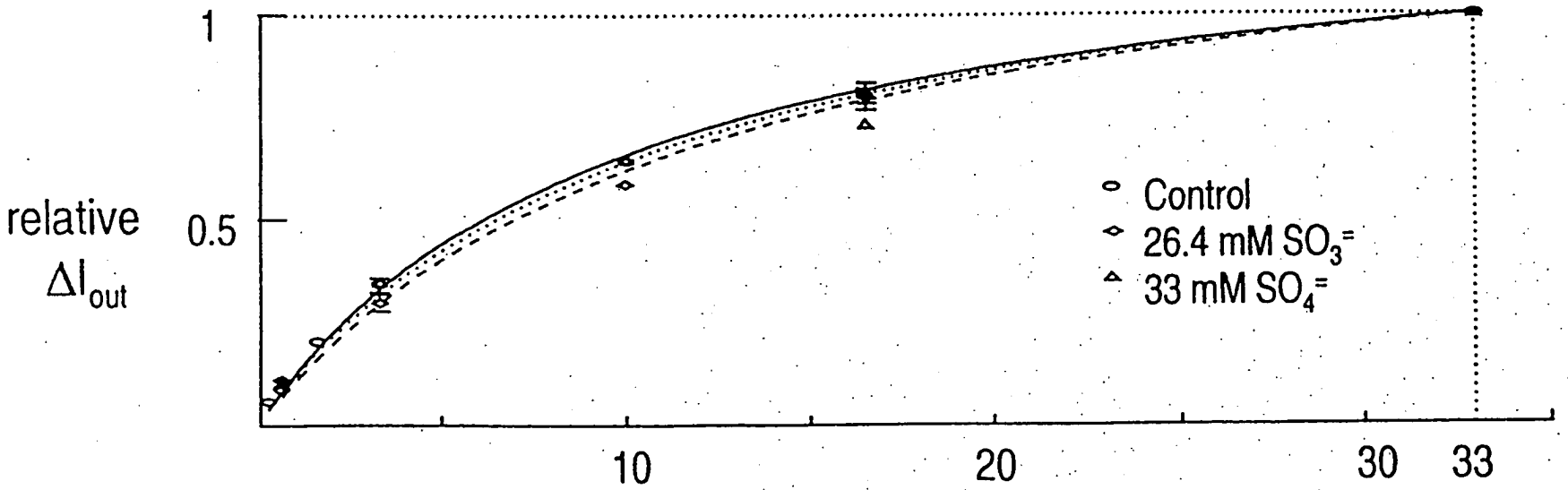
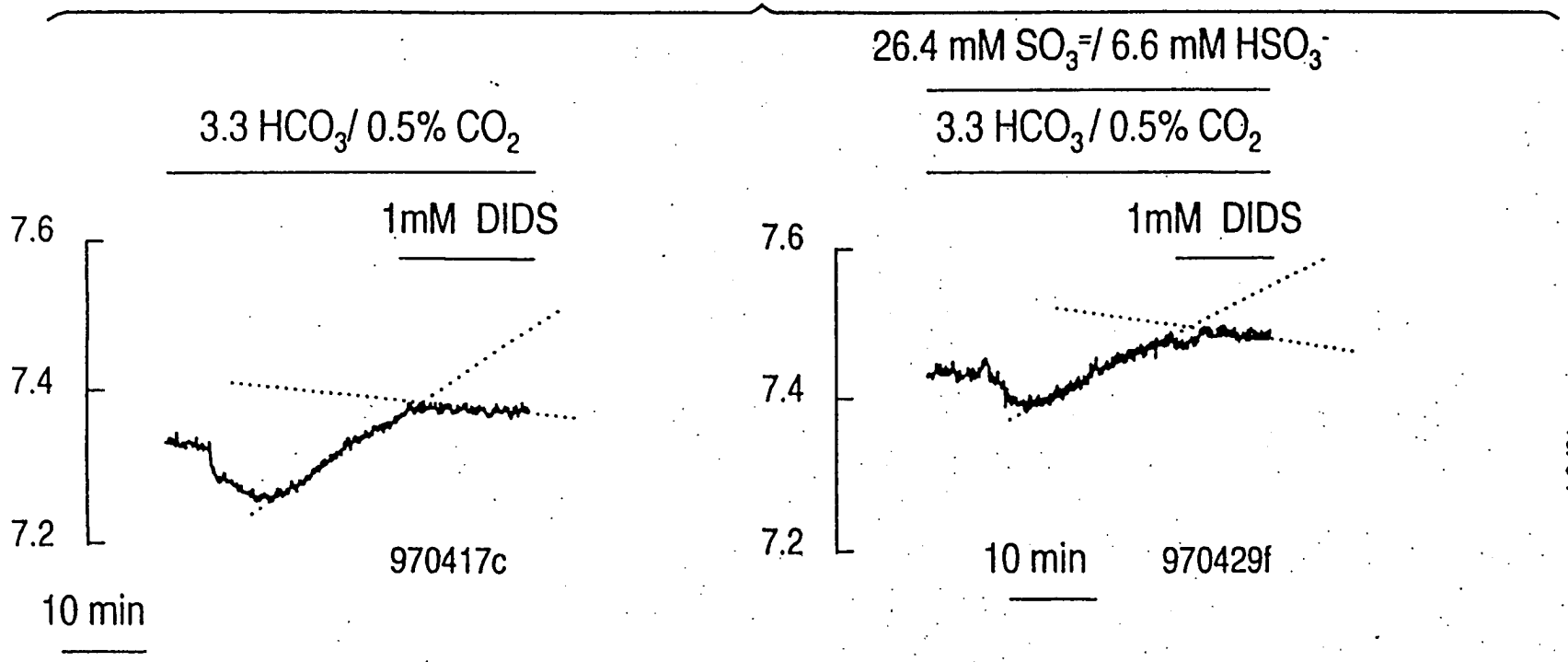



FIG. 24B



# FIG. 25




$\Delta dpH_i/dt = 0.98 \pm 0.24$
(pH unit / sec x $10^{-4}$) (n = 5)

$\Delta dpH_i/dt = 0.87 \pm 0.31$
(pH unit / sec x $10^{-4}$) (n = 6)

# FIG. 26A1

TCCAGCAGCAGCATCCTAAAACCTCTCATCTCTCCTGCTGCAGAACGCATCCGATTCATCTTGGGAGAGGAG
GATGACAGCCCAGCTCCCCCTCAGCTCTTCACGGAACTGGATGAGCTGCTGGCCGTGGATGGGCAGGAGATG
GAGTGGAAGGAAACAGCCAGGTGGATCAAGTTTGAAGAAAAAGTGGAACAGGGTGGGGAAAGATGGAGCAAG
CCCCATGTGGCCACATTGTCCCTTCATAGTTTATTTGAGCTGAGGACATGTATGGAGAAAGGATCCATCATG
CTTGATCGGGAGGCTTCTTCTCTCCCACAGTTGGTGGAGATGATTGTTGACCATCAGATTGAGACAGGCCTA
TTGAAACCTGAACTTAAGGATAAGGTGACCTATACTTTGCTCCGGAAGCACCGGCATCAAACCAAGAAATCC
AACCTTCGGTCCCTGGCTGACATTGGGAAGACAGTCTCCAGTGCAAGTAGGATGTTTACCAACCCTGATAAT
GGTAGCCCAGCCATGACCCATAGGAATCTGACTTCCTCCAGTCTGAATGACATTTCTGATAAACCGGAGAAG
GACCAGCTGAAGAATAAGTTCATGAAAAAATTGCCACGTGATGCAGAAGCTTCCAACGTGCTTGTTGGGGAG
GTTGACTTTTTGGATACTCCTTTCATTGCCTTTGTTAGGCTACAGCAGGCTGTCATGCTGGGTGCCCTGACT
GAAGTTCCTGTGCCCACAAGGTTCTTGTTCATTCTCTTAGGTCCTAAGGGGAAAGCCAAGTCCTACCACGAG
ATTGGCAGAGCCATTGCCACCCTGATGTCTGATGAGGTGTTCCATGACATTGCTTATAAAGCAAAAGACAGG
CACGACCTGATTGCTGGTATTGATGAGTTCCTAGATGAAGTCATCGTCCTTCCACCTGGGGAATGGGATCCA
GCAATTAGGATAGAGCCTCCTAAGAGTCTTCCATCCTCTGACAAAAGAAAGAATATGTACTCAGGTGGAGAG
AATGTTCAGATGAATGGGGATACGCCCCATGATGGAGGTCACGGAGGAGGAGGACATGGGGATTGTGAAGAA
TTGCAGCGAACTGGACGGTTCTGTGGTGGACTAATTAAAGACATAAAGAGGAAAGCGCCATTTTTTGCCAGT
GATTTTTATGATGCTTTAAATATTCAAGCTCTTTCGGCAATTCTCTTCATTTATCTGGCAACTGTAACTAAT
GCTATCACTTTTGGAGGACTGCTTGGGGATGCCACTGACAACATGCAGGGCGTGTTGGAGAGTTTCCTGGGC
ACTGCTGTCCCGGAGCCATTTTCTTGCCTTTTTGCTGTTCAACAATTCATTATTTTGAGCAGCACCGGACCT
GTCCTAGTTTTTGAGAGGCTTCTATTTAATTTCAGCAAGGACAATAATTTTGACTATTTGGAGTTTCGCCTT
TGGATTGGCCTGTGGTCCGCCTTCCTATGTCTCATTTTGGTAGCCACTGATGCCAGCTTCTTGGTTCAATAC

# FIG. 26A2

TTCACACGTTTCACGGAGGAGGGCTTTTCCTCTCTGATTAGCTTCATCTTTATCTATGATGCTTTCAAGAAG
ATGATCAAGCTTGCAGATTACTACCCCATCAACTCCAACTTCAAAGTGGGCTACAACACTCTCTTTTCCTGT
ACCTGTGTGCCACCTGACCCAGCTAATATCTCAATATCTAATGACACCACACTGGCCCCAGAGTATTTGCCA
ACTATGTCTTCTACTGACATGTACCATAATACTACCTTTGACTGGGCATTTTTGTCGAAGAAGGAGTGTTCA
AAATACGGAGGAAACCTCGTCGGGAACAACTGTAATTTTGTTCCTGATATCACACTCATGTCTTTTATCCTC
TTCTTGGGAACCTACACCTCTTCCATGGCTCTGAAAAAATTCAAAACTAGTCCTTATTTTCCAACCACAGCA
AGAAAACTGATCAGTGATTTTGCCATTATCTTGTCCATTCTCATCTTTTGTGTAATAGATGCCCTAGTAGGC
GTGGACACCCCAAAACTAATTGTGCCAAGTGAGTTCAAGCCAACAAGTCCAAACCGAGGTTGGTTCGTTCCA
CCGTTTGGAGAAAACCCCTGGTGGGTGTGCCTTGCTGCTGCTATCCCGGCTTTGTTGGTCACTATACTGATT
TTCATGGACCAACAAATTACAGCTGTGATTGTAAACAGGAAAGAACATAAACTCAAGAAGGGAGCAGGGTAT
CACTTGGATCTCTTTTGGGTGGCCATCCTCATGGTTATATGCTCCCTCATGGCTCTTCCGTGGTATGTAGCT
GCTACGGTCATCTCCATTGCTCACATCGACAGTTTGAAGATGGAGACAGAGACTTCTGCACCTGGAGAACAA
CCAAAGTTTCTAGGAGTGAGGGAACAAAGAGTCACTGGAACCCTTGTGTTTATTCTGACTGGTCTGTCAGTC
TTTATGGCTCCCATCTTGAAGTTTATACCCATGCCTGTACTCTATGGTGTGTTCCTGTATATGGGAGTAGCA
TCCCTTAATGGTGTGCAGTTCATGGATCGTCTGAAGCTGCTTCTGATGCCTCTGAAGCATCAGCCTGACTTC
ATCTACCTGCGTCATGTTCTTCTGCGCAGAGTCCACCTGTTCACTTTCCTGCAGGTGTTGTGTCTGGCCCTG
CTTTGGATCCTCAAGTCAACGGTGGCTGCTATCATTTTTCCAGTAATGATCTTGGCGCTTGTAGCTGTCAGA
AAAGGCATGGACTACCTCTTCTCCCAGCATGACCTCAGCTTCCTGGATGATGTCATTCCAGAAAAGGACAAG
AAAAAGAAGGAGGATGAGAAGAAAAAGAAAAAGAAGAAGGGAAGTCTGGACAGTGACAATGATGATTCTGAC
TGCCCATACTCAGAAAAAGTTCCAAGTATTAAAATTCCAATGGACATCATGGAACAGCAACCTTTCCTAAGC
GATAGCAAACCTTCTGACAGAGAAAGATCACCAACATTCCTTGAACGCCACACATCATGC

# FIG. 26B

SSSSILKPLISPAAERIRFILGEEDDSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEEKVEQGGERWSK
PHVATLSLHSLFELRTCMEKGSIMLDREASSLPQLVEMIVDHQIETGLLKPELKDKVTYTLLRKHRHQTKKS
NLRSLADIGKTVSSASRMFTNPDNGSPAMTHRNLTSSSLNDISDKPEKDQLKNKFMKKLPRDAEASNVLVGE
VDFLDTPFIAFVRLQQAVMLGALTEVPVPTRFLFILLGPKGKAKSYHEIGRAIATLMSDEVFHDIAYKAKDR
HDLIAGIDEFLDEVIVLPPGEWDPAIRIEPPKSLPSSDKRKNMYSGGENVQMNGDTPHDGGHGGGGHGDCEE
LQRTGRFCGGLIKDIKRKAPFFASDFYDALNIQALSAILFIYLATVTNAITFGGLLGDATDNMQGVLESFLG
TAVPEPFSCLFAVQQFIILSSTGPVLVFERLLFNFSKDNNFDYLEFRLWIGLWSAFLCLILVATDASFLVQY
FTRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINSNFKVGYNTLFSCTCVPPDPANISISNDTTLAPEYLP
TMSSTDMYHNTTFDWAFLSKKECSKYGGNLVGNNCNFVPDITLMSFILFLGTYTSSMALKKFKTSPYFPTTA
RKLISDFAIILSILIFCVIDALVGVDTPKLIVPSEFKPTSPNRGWFVPPFGENPWWVCLAAAAIPALLVTILI
FMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILMVICSLMALPWYVAATVISIAHIDSLKMETETSAPGEQ
PKFLGVREQRVTGTLVFILTGLSVFMAPILKFIPMPVLYGVFLYMGVASLNGVQFMDRLKLLLMPLKHQPDF
IYLRHVLLRRVHLFTFLQVLCLALLWILKSTVAAIIFPVMILALVAVRKGMDYLFSQHDLSFLDDVIPEKDK
KKKEDEKKKKKKKGSLDSDNDDSDCPYSEKVPSIKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC

# FIG. 27AI

TCCAGCAGCAGCATCCTAAAACCTCTCATCTCTCCTGCTGCAGAACGCATCCGATTCATCTTGGGAGAGGAG
GATGACAGCCCAGCTCCCCCTCAGCTCTTCACGGAACTGGATGAGCTGCTGGCCGTGGATGGGCAGGAGATG
GAGTGGAAGGAAACAGCCAGGTGGATCAAGTTTGAAGAAAAAGTGGAACAGGGTGGGGAAAGATGGAGCAAG
CCCCATGTGGCCACATTGTCCCTTCATAGTTTATTTGAGCTGAGGACATGTATGGAGAAAGGATCCATCATG
CTTGATCGGGAGGCTTCTTCTCTCCCACAGTTGGTGGAGATGATTGTTGACCATCAGATTGAGACAGGCCTA
TTGAAACCTGAACTTAAGGATAAGGTGACCTATACTTTGCTCCGGAAGCACCGGCATCAAACCAAGAAATCC
AACCTTCGGTcCCTGGCTGACATTGGGAAGACAGTCTCCAGTGCAAGTAGGATGTTTACCAACCCTGATAAT
GGTAGCCCAGCCATGACCCATAGGAATCTGACTTCCTCCAGTCTGAATGACATTTCTGATAAACCGGAGAAG
GACCAGCTGAAGAATAAGTTCATGAAAAAATTGCCACGTGATGCAGAAGCTTCCAACGTGCTTGTTGGGGAG
GTTGACTTTTTGGATACTCCTTTCATTGCCTTTGTTAGGCTACAGCAGTCTGTCATGCTGGGTGCCCTGACT
GAAGTTCCTGTGCCCACAAGGTTCTTGTTCATTCTCTTAGGTCCTAAGGGGAAAGCCAAGTCCTACCACGAG
ATTGGCAGAGCCATTGCCACCCTGATGTCTGATGAGGTGTTCCATGACATTGCTTATAAAGCAAAAGACAGG
CACGACCTGATTGCTGGTATTGATGAGTTCCTAGATGAAGTCATCGTCCTTCCACCTGGGGAATGGGATCCA
GCAATTAGGATAGAGCCTCCTAAGAgTCTTCCATCCTCTGACAAAAGAAAGAATATGTACTCAGGTGGAGAG
AATGTTCAGATGAATGGGGATACGCCCCATGATGGAGGTCACGGAGGAGGAGGACATGGGGATTGTGAAGAA
TTGCAGCGAACTGGACGGTTCTGTGGTGGACTAATTAAAGACATAAAGAGGAAAGCGCCATTTTTTGCCAGT
GATTTTTATGATGCTTTAAATATTCAAGCTCTTTCGGCAATTCTCTTCATTTATCTGGCAACTGTAACTAAT
GCTATCACTTTTGGAGGACTGCTTGGGGATGCCACTGACAACATGCAGGGCGTGTTGGAGAGTTTCCTGGGC
ACTGCTGTCTCTGGAGCCATCTTTTGCATTTTTGCTGGTCAACCACTCACTATTCTGAGCAGCACCGGACCT
GTCCTAGTTTTTGAGAGGCTTCTATTTAATTTCAGCAAGGACAATAATTTTGACTATTTGGAGTTTCGCCTT
TGGATTGGCCTGTGGTCCGCCTTCCTATGTCTCATTTTGGTAGCCACTGATGCCAGCTTCTTGGTTCAATAC

# FIG. 27A2

TTCACACGTTTCACGGAGGAGGGCTTTTCCTCTCTGATTAGCTTCATCTTTATCTATGATGCTTTCAAGAAG
ATGATCAAGCTTGCAGATTACTACCCCATCAACTCCAACTTCAAAGTGGGCTACAACACTCTCTTTTCCTGT
ACCTGTATGCCACCTGACCCAGCTAATATCTCAATATCTAATGACACCACACTGGCCCCAGAGTATTTGCCA
ACTATGTCTTCTACTGACATGTACCATAATACTACCTTTGACTGGGCATTTTTGTCGAAGAAGGAGTGTTCA
AAATACGGAGGAAACCTCGTCGGGAACAACTGTAATTTTGTTCCTGATATCACACTCATGTCTTTTATCCTC
TTCTTGGGAACCTACACCTCTTCCATGGCTCTGAAAAAATTCAAAACTAGTCCTTATTTTCCAACCACAGCA
AGAAAACTGATCAGTGATTTTGCCATTATCTTGTCCATTCTCATCTTTTGTGTAATAGATGCCCTAGTAGGC
GTGGACACCCCAAAACTAATTGTGCCAAGTGAGTTCAAGCCAACAAGTCCAAACCGAGGTTgGTTCGTTCCA
CCGTTTGGAGAAAACCCCTGGTGGGTGTGCCTTGCTGCTGCTATCCCGGCTTTGTTGGTCACTATACTGATT
TTCATGGACCAACAAATTACCGCTGTGATTGTAAACAGGAAAGAACATAAACTCAAGAAAGGAGCAGGGTAT
CACTTGGATCTCTTTTGGGTGGCCATCCTCATGGTTATATGCTCCCTCATGGCTCTTCCGTGGTATGTAGCT
GCTACGGTCATCTCCATTGCTCACATCGACAGTTTGAAGATGGAGACAGAGACTTCTGCACCTGGAGAACAA
CCAAAGTTTCTAGGAGTGAGGGAACAAAGAGTCACTGGAACCCTTGTGTTTATTCTGACTGGTCTGTCAGTC
TTTATGGCTCCCATCTTGAAGTTTATACCCATGCCTGTACTCTATGGTGTGTTCCTGTATATGGGAGTAGCA
TCCCTTAATGGTGTGCAGTTCATGGATCGTCTGAAGCTGCTTCTGATGCCTCTGAAGCATCAGCCTGAcTTC
ATCTACCTGCGTCATGTTCCTCTGCGCAGAGTCCACCTGTTCACTTTCCTGCAGGTGTTGTGTCTGGCCCTG
CTTTGGATCCTCAAGGCAACGGTGGCTGCTATCATTTTTCCAGTAATGATCTTGGCACTTGTAGCTGTCAGA
AAAGGCATGGACTACCTCTTCTCCCAGCATGACCTCAGCTTCCTGGATGATGTCATTCCAGAAAAGGACAAG
AAAAAGAAGGAGGATGAGAAGAAAAAGAAAAAGAAGAAGGGAAGTCTGGACAGTGACAATGATGATTCTGAC
TGCCCATACTCAGAAAAAGTTCCAAGTATTAAAATTCCAATGGACATCATGGAACAGCAACCTTTCCTAAGC
GATAGCAAACCTTCTGACAGAGAAAGATCACCAACATTCCTTGAACGCCACACATCATGCTAA

# FIG. 27B

SSSSILKPLISPAAERIRFILGEEDDSPAPPQLFTELDELLAVDGQEMEWKETARWIKFEEKVEQGGERWSK
PHVATLSLHSLFELRTCMEKGSIMLDREASSLPQLVEMIVDHQIETGLLKPELKDKVTYTLLRKHRHQTKKS
NLRSLADIGKTVSSASRMFTNPDNGSPAMTHRNLTSSSLNDISDKPEKDQLKNKFMKKLPRDAEASNVLVGE
VDFLDTPFIAFVRLQQSVMLGALTEVPVPTRFLFILLGPKGKAKSYHEIGRAIATLMSDEVFHDIAYKAKDR
HDLIAGIDEFLDEVIVLPPGEWDPAIRIEPPKSLPSSDKRKNMYSGGENVQMNGDTPHDGGHGGGGHGDCEE
LQRTGRFCGGLIKDIKRKAPFFASDFYDALNIQALSAILFIYLATVTNAITFGGLLGDATDNMQGVLESFLG
TAVSGAIFCIFAGQPLTILSSTGPVLVFERLLFNFSKDNNFDYLEFRLWIGLWSAFLCLILVATDASFLVQY
FTRFTEEGFSSLISFIFIYDAFKKMIKLADYYPINSNFKVGYNTLFSCTCMPPDPANISISNDTTLAPEYLP
TMSSTDMYHNTTFDWAFLSKKECSKYGGNLVGNNCNFVPDITLMSFILFLGTYTSSMALKKFKTSPYFPTTA
RKLISDFAIILSILIFCVIDALVGVDTPKLIVPSEFKPTSPNRGWFVPPFGENPWWVCLAAAIPALLVTILI
FMDQQITAVIVNRKEHKLKKGAGYHLDLFWVAILMVICSLMALPWYVAATVISIAHIDSLKMETETSAPGEQ
PKFLGVREQRVTGTLVFILTGLSVFMAPILKFIPMPVLYGVFLYMGVASLNGVQFMDRLKLLLMPLKHQPDF
IYLRHVPLRRVHLFTFLQVLCLALLWILKATVAAIIFPVMILALVAVRKGMDYLFSQHDLSFLDDVIPEKDK
KKKEDEKKKKKKKGSLDSDNDDSDCPYSEKVPSIKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC

# INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 98/10297

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K14/46 C12N5/10 C07K16/18
A61K48/00 A61K39/395 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BORON V F ET AL: "The renal electrogenic Na +:HCO-3 cotransporter." JOURNAL OF EXPERIMENTAL BIOLOGY, (1997 JAN) 200 ( PT 2) 263-8. REF: 24 JOURNAL CODE: I2F. ISSN: 0022-0949., XP002076892 ENGLAND: United Kingdom see abstract see page 265, right-hand column, paragraph 4 - page 267, right-hand column, last paragraph --- -/-- | 1-24 |

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 October 1998 | 16/10/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Montero Lopez, B |

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# INTERNATIONAL SEARCH REPORT

Internat al Application No
PCT/US 98/10297

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | ROMERO M F ET AL: "Cloning and functional expression of the rat renal electrogenic Na - HCO3 cotransporter (rNBC)." 29TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF NEPHROLOGY, NEW ORLEANS, LOUISIANA, USA, NOVEMBER 3-6, 1996. JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY 7 (9). 1996. 1259. ISSN: 1046-6673, XP002076893 see abstract no. A0055 | 1-9 |
| P,X | CHARLES E. BURNHAM ET AL.: "Cloning and functional expression of a human kidney Na+:HCO3- Cotransporter" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 31, 1 August 1997, pages 19111-19114, XP002076894 MD US see the whole document | 1-24 |
| P,X | ROMERO M F ET AL: "Cloning and functional expression of rNBC, an electrogenic Na (+)- HCO3 - cotransporter from rat kidney." AMERICAN JOURNAL OF PHYSIOLOGY, (1998 FEB) 274 (2 PT 2) F425-32. JOURNAL CODE: 3U8. ISSN: 0002-9513., XP002076895 United States see abstract see page F427, left-hand column, paragraph 3 - page F431, left-hand column, paragraph 3 | 1-24 |
| P,X | MICHAEL F. ROMERO ET AL.: "Expression cloning and characterization of a renal electrogenic Na+/HCO3- cotransporter" NATURE, vol. 387, 22 May 1997, pages 409-413, XP002076896 LONDON GB see the whole document | 1-24 |
| P,X | Trrod Database Entry 035422 Accession number 035422; 1 January 1998 BURNHAM C.E. ET AL. XP002076897 see the whole document | 9-11 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 10297

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 12-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 98/10297

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K14/46 C12N5/10 C07K16/18
A61K48/00 A61K39/395 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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# INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/10297

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**Remark on Protest**

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[ ] No protest accompanied the payment of additional search fees.